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A Simple Method for the Urinary Folic Acid Assay in Folic Acid Load Test

Studies on the Nutrition of Children in Hirosaki Area

(48th Report)

By

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Cases of macrocytic anemia, which responded to folic acid,[1),2)] were found not infrequently among children with nutritional dystrophy in the Hirosaki area. For the purpose of investigating the relationship between folic acid metabolism and macrocytic anemia, a simple and rapid method for the determination of pteroylglutamic acid (PGA) excreted in the urine after a test dose of PGA is described in the present paper.

Method

(A) Collection of urine specimens

Specimens I and II are obtained: Specimen I is a specimen of urine voided and collected for 24 hours just prior to the day when the test dose of PGA will be given orally, and Specimen II is that voided and collected for 24 hours following the oral administration of that test dose or of 10 mg. of PGA.

Specimen I is used for the preparation of the standard curve (peculiar to the respective case).

(B) Fluorometric estimation of PGA in urine

(i) To 1.0 cc. of Specimen II add 4.0 cc. of 10% lead acetate solution and keep the mixture in a water bath at 60°C. for 30 minites with stirring for about 5 minutes. After centrifugation, wash the precipitate twice with 4 cc. of 10% lead acetate. To the precipitate add 4 cc. of 10% ammonium sulfate and keep the content in a water bath at 60°C. for 20 minutes with stirring for 3 minutes, and centrifuge. Transfer 2.0 cc. of the upper clear layer into a test tube, marked at 10 cc., add 0.1 cc. of 2.5 *M* acetate buffer of pH 4, then 0.2 cc. of 4% potassium permanganate, and mix the content vigorously for a few minutes, and let stand for 5 minutes at room temperature. Then add 1 cc. of 3% H_2O_2 and mix

the content, (if the color of potassium permanganate has not disappeared, more H_2O_2 will be added), and make up to the mark by the addition of distilled water. On the colorless solution thus obtained, the intensity of fluorescence is measured by means of Yagi's fluorometer.[3]

(ii) Preparation of the standard curve

Using 1.0 cc. of Specimen I, precipitation with lead acetate, elution in ammonium sulfate and oxidation by permanganate are carried out in the same way as described above (i). The reading of the fluorometer is plotted on the ordinate and through that point draw a line (B) parallel to Line F in Fig. 1 (Cf. Fig. 1). This Line F has been obtained from the

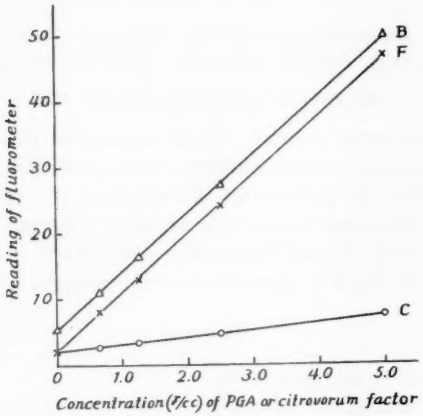

Fig. 1. The concentrations of PGA or citrovorum factor and their relation to the intensity of the fluorescence determined by My own method.

fluorometric assay as described first (i) from the respective standard solutions of PGA, viz. the solutions of 0.625, 1.250 and 2.500 γ of PGA, each in 1.0 cc. of $M/15$ KH_2PO_4 solution. Line B is used for the standard curve, by which PGA concentration in Specimen II of the case in question can be calculated.

(iii) Fluorometer

Yagi's fluorometer,[3] equipped with the primary filter, transmitting at 365 mμ, and with the secondary filter, transmitting at 470 mμ, was used throughout this investigation, and the sensitivity of the apparatus was adjusted at '5' degree.

Discussion

According to Wittle *et al.*,[4] permanganate oxidation of PGA yielded a strong fluorescent substance, identified as 2-amino-4-hydroxypteridine-

6-carboxylic acid, and the intensity of fluorescence was directly proportional to PGA concentration. According to Allfrey *et al.*,[5] in the fluorometric determination of PGA in tissue extract, the interfering substances were eliminated by the following procedures: the oxidation product of PGA was adsorbed on Frorisil, then eluated with sodium tetraborate solution. Fluorometric reading was done before and after the addition of 40% NaOH to the solution containing the oxidation product of PGA, because the fluorescence arising from the PGA derivative disappeared almost completely in strong alkaline solution.

The present investigation of mine was attempted to determine a relatively large amount of PGA in urine after oral ingestion of 10 mg. of PGA.

My own modification differed in the following two points from the original method of Allfrey *et al.*: (1) the fluorometric reading after the addition of 40% NaOH was omitted, because in the majority of cases, the intensity of fluorescence of Specimen I did not decrease, but slightly increased after the addition of 40% NaOH, showing that the fluorescence in Specimen I was not due to the PGA derivative (Cf. Table II). (2) Further, in my own modification, PGA was precipitated from urine with lead acetate and eluated with ammonium sulfate solution,[6] while in the original method, the oxidation product of PGA was adsorbed on Frorisil and eluated with sodium tetraborate solution.

As will be seen from Table I and Fig. 1, in my own method, the intensity of the fluorescence arising from PGA was found to be proportional

TABLE I

The Intensity of Fluorescence from PGA or Citrovorum Factor in My Method

PGA		PGA+Urine		Citrovorum factor	
PGA (γ/cc.) dissolved in M/15 KH_2PO_4 solution	Intensity of the fluorescence	PGA (γ/cc.) added to the urine	Intensity of the fluorescence	Citrovorum factor (γ/cc. dissolved in M/15 KH_2PO_4 solution	Intensity of the fluorescence
0	2.2	0	5.2	0	2.2
0.625	8.0	0.625	11.1	0.625	2.8
1.250	13.0	1.250	16.2	1.250	3.1
2.500	24.0	2.500	27.0	2.500	4.5
5.000	47.0	5.000	50.1	5.000	7.5

to the concentration of PGA, whether PGA was dissolved in *M*/15 KH_2PO_4 solution or in urine. So Line B ran parallel to Line F as shown in Fig. 1.

TABLE II

Change in the Intensity of Fluorescence before and after the Addition of 40% NaOH

No. of urine specimens	Fluorometric reading	
	The addition of NaOH	
	Before	After
1	5.5	5.0
2	4	6.2
3	6	9.0
4	8	8.8
5	3	6.5
6	3	3
7	2	3
8	2.2	4
9	2	4.5
10	2	2.5
11	4.5	6

When the citrovorum factor, dissolved in $M/15$ KH_2PO_4 solution, was treated in the same way as described above (i), the intensity of fluorescence arisen from citrovorum factor was far less than that from PGA of the equivalent concentration (Cf. Table I, further Lines F and C in Fig. 1).

Summary and Conclusions

A simple and rapid method, a modification of Allfrey *et al.*'s, was described for the determination of PGA excreted in the urine after oral ingestion of PGA. In this modification of my own, PGA excreted in the urine was precipitated by lead acetate and eluated in ammonium sulfate, then oxidized by permanganate according to Allfrey *et al.*'s principle, excepting the fluorometric reading after the addition of NaOH to the oxidation product of PGA. The PGA added to urine was recovered satisfactorily in my modified method.

References

1) Arakawa, Wada & Takahashi, Tohoku J. Exp. Med., 1951, **55**, 61.
2) Wada & Arakawa, *ibid.*, 1952, **56**, 47.
3) Yagi, Recent Methods for Vitamin Assay (Jap.), 1st ed., Ishyaku Schuppan., Tokyo, 1954.
4) Wittle, O'Dell, Vandenbelt & Pfiffner, J. Am. Chem. Soc., 1947, **69**, 1786.
5) Allfrey, Jeply, Geffen & King, J. Biol. Chem., 1949, **178**, 465.
6) Robinson, The Vitamin B Complex, 1st ed., 1951, p. 467, Chapman & Hall, London.

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Folic Acid Deficiency in Children with Nutritional Dystrophy

Studies on the Nutrition of Children in Hirosaki Area

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Among the children with nutritional dystrophy in this District, macrocytic anemia was not infrequently found.[1] And such cases with the anemia responded sharply to folic acid.[2] In the present paper, the results of the folic acid load test performed upon children with or without macrocytic anemia will be discussed.

Experimental

Method and Material

The children subjected to this investigation were divided into 2 groups (Groups I and II); Group I included 6 boys who were healthy and free from ariboflavinotic signs,[3] and Group II included 19 boys who showed marked signs of ariboflavinosis as part manifestations of nutritional dystrophy.

The blood picture was examined just prior to the folic acid load test, and according to the mean corpuscular volume, the term of macrocytosis ($>95\ \mu^3$) or microcytosis ($<79\ \mu^3$) was used.[4]

Folic acid load test was carried out as described in my preceding paper[5]; pterolylglutamic acid excreted in the 24 hour-urine following oral ingestion of 10 mg. of pterolylglutamic acid was determined fluorometrically by means of my own procedure.[5]

Results with Discussion

The results were shown in Tables I and II (Cf. Tables I and II). PGA excreted in the 24 hours urine following oral dose of 10 mg. of PGA was found to be 4.29 ± 0.61 mg. and 2.36 ± 0.81 mg. in Groups I and II respectively, showing a significant difference between them (Cf. Tables

TABLE I

Blood Picture and Folic Acid Excreted in the 24 Hour Urine Following Oral Ingestion of 10 mg. of Folic Acid in Children without Nutritional Dystrophy (Group I)

No. of Case	Name, Sex and Age (Years and months)	The 24 hour urine		Blood picture			
		Amount cc.	Folic acid mg.	Red cells (millions)	Hemoglobin (g%)	Hematocrit (%)	Mean corpuscular volume (μ^3)
1	K.M. ♂ 10y.7m.	1020	5.11	4.90	12.4	46	93
2	M.K. ♂ 11y.1m.	1060	4.17	4.90	12.4	45	91
3	K.K. ♂ 11y.6m.	620	3.16	4.80	11.6	47	97
4	K.H. ♂ 12y.3m.	1130	4.86	4.30	10.4	40	93
5	S.Y. ♂ 11y.5m.	1250	3.68	5.10	11.2	45	88
6	K.Y. ♂ 12y. 2m.	1380	4.29	4.50	10.8	41	91
	Average		4.29 ± 0.61	4.75 ± 0.31	11.4 ± 1.6	44 ± 2.9	92 ± 7.1

I and II).

On the other hand macrocytic anemia was found in 16 out of 19 cases of Group II—these 16 cases responded sharply to folic acid[2]—, but in none of Group I (Cf. Tables I and II).

These two findings—a low excretion of PGA after oral test dose as well as a high incidence in macrocytic anemia in the children with nutritional dystrophy,—might be accepted as an evidence for the existence of folic acid deficiency in these children.

According to the general literature, anyone of the following 4 factors would contribute to the low excretion of PGA after its oral dose; a deficient intake of PGA, an increased requirement of it, the conjugation of it before excretion[6] and malabsorption from the intestine.[7] Stepp *et al.*[8] described that folic acid deficiency, which would manifeste itself as in the form of macrocytic anemia as well as in the form of intestinal disturbance, was observed in cases of malnutrition, and that folic acid deficiency occurred, in general, not from a deficient intake of folic acid itself, but from protein deficiency accompanied by malnutrition, because protein deficiency brought out both an increased requirement for folic acid and a depression of the synthesis of folic acid by intestinal flora.

TABLE II

Blood Picture and Folic Acid Excreted in the 24 Hour Urine Following Oral Ingestion of 10 mg. of Folic Acid in Children with Nutritional Dystrophy (Group II)

No. of Case	Name, Sex and Age (Years and months)	The 24 hour urine		Blood picture			
		Amount cc.	Folic acid mg.	Red cells (millions)	Hemoglobin (g%)	Hematocrit (%)	Mean corpuscular (μ^3)
1	H.S. ♂ 10y.1m.	1260	1.78	4.10	7.50	42.0	102
2	H.M. ♂ 9y.10m.	1440	5.88	4.30	9.30	43.0	100
3	K.A. ♂ 9y.8m.	1560	2.88	3.85	8.20	39.0	101
4	K.Z. ♂ 9y.3m.	2320	0	4.10	8.90	41.0	100
5	T.R. ♂ 9y.1m.	1020	1.65	3.80	7.90	37.0	97
6	T.O. ♂ 8y.11m.	640	3.66	3.80	7.90	35.0	92
7	H.S. ♂ 8y.11m.	1260	1.62	3.85	8.90	37.0	96
8	T.T. ♂ 8y.9m.	1000	1.69	3.85	7.90	36.0	93
9	K.K. ♂ 8y.8m.	1400	1.27	3.65	7.50	38.0	104
10	K.M. ♂ 8y.8m.	820	3.12	3.20	7.50	37.0	115
11	I.M. ♂ 8y.7m.	1180	1.55	3.85	8.20	40.0	103
12	K.K. ♂ 8y.5m.	1360	0.52	3.90	7.90	41.0	105
13	M.M. ♂ 8y.5m.	1600	0.42	3.90	6.80	36.0	92
14	I.K. ♂ 8y.5m.	1440	2.33	3.50	6.70	36.0	102
15	N.S. ♂ 8y.5m.	1160	2.28	3.90	7.50	38.0	97
16	S.M. ♂ 8y.5m.	1800	2.85	3.80	7.50	39.0	102
17	S.E. ♂ 8y.4m.	1300	0.92	3.75	6.40	37.0	98
18	W.T. ♂ 8y.4m.	1080	6.01	3.85	7.50	39.0	101
19	K.S. ♂ 8y.4m.	1400	4.46	3.50	7.90	42.0	120
	Average		2.36 ± 0.81	3.81 ± 0.14	7.78 ± 0.39	38.5 ± 1.7	101 ± 3.7

In cases of macrocytic anemia of children with nutritional dystrophy in Hirosaki area, a gradual improvement of blood picture was observed after a continuous administration of skimmed milk for 3 months[9] without any dose of folic acid. So protein deficiency might play an important rôle for the development of folic acid deficiency in these children.

As will be seen from Fig. 1 (Cf. Fig. 1), a relatively high value of the PGA excreted after its oral dose was seen in 3 cases (Cases 2, 18 and 19) in which macrocytic anemia had been demonstrated. This apparent

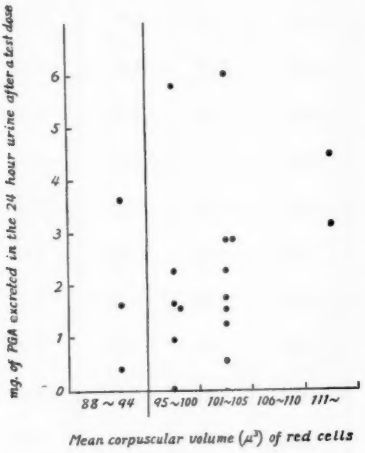

Fig. 1. The folic acid excretion in the 24-hour urine following oral ingestion of 10 mg. of folic acid and mean corpuscular volume of red cells in children with nutritional dystrophy.

discrepancy might be well explained, if the following items are taken into consideration: (1) hepatic changes have been not infrequently demonstrated in the liver specimens biopsied from children with nutritional dystrophy,[10] (2) macrocytic anemia has been found with a high incidence among those children with nutritional dystrophy whose liver has shown histological alterations in the biopsied specimens,[11] and (3) in hepatic diseases, according to Inoue,[12] a relatively large amount of folic acid, not citrovorum factor, is excreted into urine after subcutaneous injection of 10 mg. of folic acid due to a disturbance of utilization of folic acid.

Summary

Macrocytic anemia was found in 16 out of 19 cases of children with nutritional dystrophy.

PGA excretion in the 24-hour urine after oral dose of 10 mg. of PGA

was lower in the majority of the children with nutritional dystrophy than in normal subjects, but in a few cases with macrocytic anemia, a relatively high value of the PGA excreted after the test dose was found. In these cases a disturbed utilization of folic acid due to hepatic changes of these children was suggested.

Conclusions

Folic acid deficiency will occur in the children with nutritional dystrophy. The occurrence is highly probable from the low excretion of PGA after its oral dose as well as the high incidence in macrocytic anemia among these children.

References

1) Arakawa, Wada & Takahashi, Tohoku J. Exp. Med., 1951, **55**, 61.
2) Wada & Arakawa, *ibid.*, 1952, **56**, 47.
3) Youmans, J. Am. Med. Ass., 1950, **144**, 307.
4) Wintrobe, Clinical Hematology, 3nd ed., 1952, p. 101, Lea & Febiger, Philadelphia.
5) Ooi, A Simple Method for the Urinary Folic Acid Assay, applicable to Folic Acid Load Test, this Journal, in press.
6) Steinkamp, Shukers, Totter & Day, Proc. Soc. Exp. Biol. & Med., 1946, **63**, 556.
7) Girdwood, Lancet, 1953, No. 6776, 53.
8) Stepp, Kühnau & Sohroeder, Die Vitamine und ihre klinische Anwendung, 2nd ed., 1952, 510, Ferdinand Enke Verlag, Stuttgart.
9) Wada, Hirosaki Igaku (Jap.), 1955, **6**, 232.
10) Sato, Tohoku J. Exp. Med., 1955, **63**, 93.
11) Wada & Sato, *ibid.*, 1952, **56**, 259.
12) Inoue, Vitamins (Jap.), 1956, **10**, 75.

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Age-adjusted Death Rates for Cirrhosis of Liver in 24 Countries

By

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We have computed the age-adjusted death rates for cirrhosis of liver in some recent years of 24 countries, whose central offices of vital statistics have kindly provided us with data on the number of deaths from this disease. The standard population applied in the calculation of adjustment was that of Japan by sex and age in 1953. The results are shown in Table I. Among these countries, Portugal, France, Chile and Italy belong to the countries of high rates both in males and females.

TABLE I

Age-adjusted Death Rates for Cirrhosis of Liver in 24 Countries, 1952–1954

(Rates per 100,000 population, using as standard population that of Japan in 1953)

	Male	Female		Male	Female
South Africa (White)*	9.4	4.5	Ireland	1.6	0.9
Canada	4.5	2.9	Italy*	14.0	5.5
Chile‡	20.9	9.6	Norway	2.4	1.9
U.S.A. (White)*	9.6	5.1	Netherlands	2.5	2.0
U.S.A. (Nonwhite)*	7.8	5.1	Portugal	21.5	10.4
Israel	4.7	2.9	England and Wales	1.8	1.3
Japan	9.5	6.2	Scotland	2.6	1.7
Germany, Fed. Rep.†	8.1	4.0	Northern Ireland	1.5	1.6
Austria	10.5	3.8	Sweden*	2.1	1.9
Belgium§	5.2	2.8	Switzerland*	11.8	3.1
Denmark	3.5	4.2	Australia	4.3	2.2
Finland	2.7	1.7	New Zealand	2.2	1.5
France	21.1	11.6			

* 1951–53, † 1952, 53, ‡ 1953, § 1954

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Influence of Starvation on Fatty Liver

By

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Recently, the clinical significance of fatty liver, especially the relationship between liver cirrhosis and fatty liver, has received considerable attention in Japan. We have studied fatty liver cirrhosis in albino rats fed choline-deficient diets with the aim of developing methods for treatment.[1] In these experiments fat decreased as fibrosis and ceroid accumulation progressed. Liver fat also decreased in rats which died after long periods of starvation. These findings suggested that the changes leading to fatty liver may be readily reversible. In the following experiments, an attempt was made to evaluate the influences of starvation on fatty liver.

Experimental

Material and Methods

The composition of the choline-deficient diet was as follows: 8% casein; 48% sucrose; 3% lard; 2% cod liver oil; 4% Osbornemendel's salt mixture; vitamins (per day), Vitamin B_1 50 γ, Vitamin B_2 50 γ, pyridoxine 20 γ, calcium pantothenate 100 γ, nicotinamide 500 γ, and α-tocopherol 10 mg. (per week). Two groups of eleven and twelve male albino rats with an average body weight of 146 g. and 165 g. were fed the choline deficient diet for 21 and 40 days, and then half of each group were killed as controls and the other were starved.

Liver fat was determined by the method described by Kumakawa and Sudo. Histological examination was perofrmed using Sudan III, Hematoxylin-Eosin and van Gieson stains.

Results

The curves of average body weight of the two groups are illustrated in Fig. 1. Body weight was constant for both groups while they were

receiving the choline-deficient diet. Average weekly food intake is shown in Fig. 2. A tendency towards decreased food intake was seen in both groups. The animals which were starved died an average of 15.2 days and 10.8 days after withholding food. Average body weight at the time of death was 71 g. and 101 g.

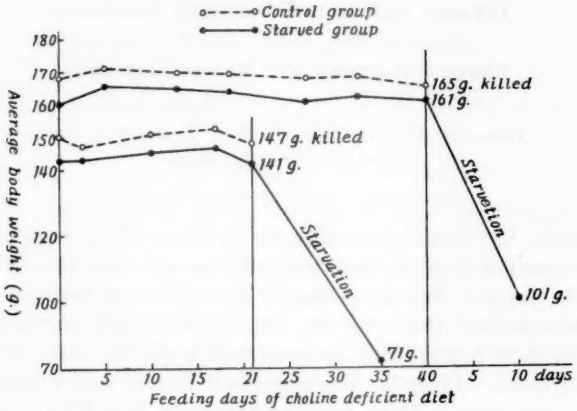

Fig. 1. The curves of average body weight of rats.

A remarkable decrease in liver fat content was observed in the starved animals (Fig. 3). Liver fat in the control groups averaged 16.3% and 22.1% (wet) but in the starved groups was only 4.6% and 5.5% (average wet). Thus, liver fat content of the starved groups was approximately $\frac{1}{4}$ that of the control groups.

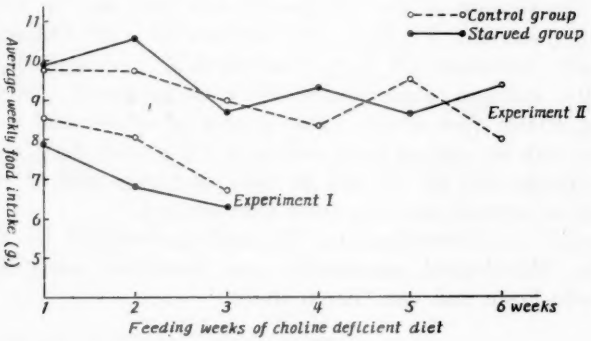

Fig. 2. Average weekly food intake of rats.

In the control group fed a choline-deficient diet for 21 days moderately

fatty livers were seen. In controls fed the choline dificient diet for 40 days markedly fatty livers were seen which were enlarged and showed yellow and white discoloration. Remarkable centrolobular fatty metamosphosis was seen in Sudan III stained sections, but liver fibrosis was not observed in either control group. In both starvation groups all livers showed a marked shrinkage. In many of the livers in both starvation groups fatty infiltration could not be seen histologically but in some of the livers in the group fed a choline-deficient diet for 40 days before starvation, relatively large fatty cysts were occasionally seen in Sudan III stained sections.

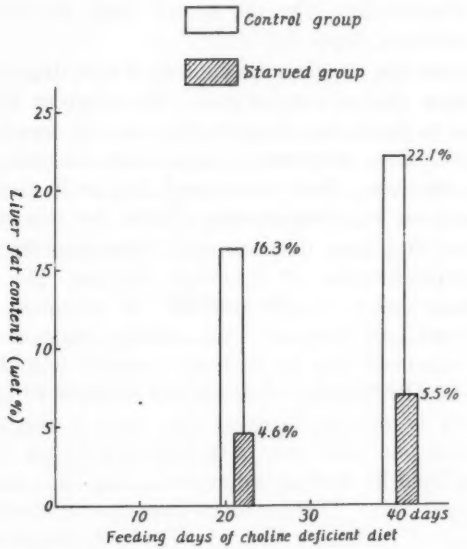

Fig. 3. Liver fat content of rats.

Discussion

Reports concerning changes of liver fat in starvation are difficult to compare, for the experimental conditions, such as species, sex, age and nutrition of the experimental animals, and factors such as temperature vary from one report to another. The fatty infiltration of the liver induced by anterior pituitary extracts has been placed by some observers in the same category as dietary fatty liver; but probably it should be classed with the fatty liver of starvation. Hypophysectomy has, however, afforded protection from the fat mobilization of starvation. The degree of fat infiltration in the liver of starved rats is dependent upon the quantity of depot fat available for mobilization. Fatty infiltration first appears at

the periphery of the liver lobule, about twenty-four hours after onset of starvation. Liver fat content reaches an average of 17% of wet weight (maximum 29%) after one to three days of starvation. When the starvation period is prolonged, neutral fat in the liver is so depleted that carcass storage lipids are mobilized. After storage fats are consumed completely, fat in liver cells is metabolized and liver cell fat content decreases to less than normal levels after four to seven days of starvation. After the neutral fat in liver is completely consumed, the phospholipids in liver decrease and then the animals die. Hodge[2] reported that mouse liver metabolized 92% of depot fat on the first day of fasting; the balance (8%) remains as sudanophil neutral fat. On the second day, the liver metabolizes 100% of the remaining depot fat.

In our experiments, the liver fat content of rats dying from starvation was much less than that of normal rats. The stage of fatty liver, which appears after one to three days of starvation was not seen because the rats survived for 9 to 11 days after onset of starvation, allowing time for metabalism of fat in the liver. Best[3] recognized that fat in the liver cells could be made to disappear by administering choline for several days.

Ueda[4] stated that fatty livers rapidly soften and shrink after one or two weeks of administration of lipotropic factors. He concluded that fatty liver cirrhosis shows a rapid response to treatment with lipotropic substances, but non-fatty liver cirrhosis exhibits resistance to such treatment. Cayer[5] observed that in cirrhotic patients with fatty infiltration of the liver (proven by biopsy), a significant increase in the rate of phospholipid turnover is usually demonstrable after a single large dose of choline or methoinine. We also demonstrated rapid and remarkable disappearance of lipid by feeding a diet containing 30% casein and 40 mg. choline chloride per day. Three cases of fatty liver cirrhosis recently autopsied in the Dept. of Pathology had been clinically diagnosed as leukemia, Weil's disease and possible liver tumor. These mistaken clinical diagnoses may be attributed to the fact that none of these patients had typical symtoms and signs of liver cirrhosis before their deaths, which occured shortly after admission to the hospital. Hartroft[6] said that in the fatty liver, fatty cysts rupture, allowing fat to escape out of the liver and then the remaining cysts atrophy and liver cirrhosis may develops by formation of collagenous strands of reticulum fibers surrounding the liver cells. Thus, liver fat content decreases and then fibrosis follows. In our experiments dealing with fatty liver cirrhosis, a decrease of liver fat content was observed in rats which died from starvation or from severe liver fibrosis. These experimental results led as to postulate that in patients having fatty cirrhosis, anorexia causes relative starvation, thereby leading to a gradual reduction in liver fat along with development of fibrosis and accumulation of ceroid.

Amano[7] emphasized that most cases of liver cirrhosis in Japan develop from infectious hepatitis, whereas fatty liver cirrhosis is very rare. However, our clinical experience indicates that fatty liver cirrhosis in Japan is not so rare. Ueda[4] has stated that finding liver cirrhosis without fatty infiltration at necropsy does not mean that fatty liver was not present in the past. The development of fatty liver may be readily and rapidly influenced by starvation, lipotropic factors, temperature and diet etc. Thus, the characteristic changes of fatty liver cirrhosis may quickly disappear and this should be born in mind in considering the pathogenesis of cirrhosis of the liver.

Summary

Male albino rats were fed a choline deficient diet for twenty-one and forty days. Half of the rats were killed as controls, and the other half were starved. Death occured after eleven to fifteen days of starvation. Liver fat of starved animals decreased markedly to approximately $\frac{1}{4}$ that of controls. These findings suggest that fatty infiltration as encountered in both experimental and necropsy specimens, may decrease or disappear rapidly due to the influence of starvation.

References

1) Nakamura, T., Nakamura, S., Sugawara, Katakura, Isono & Kawamura, Tohoku J. Exp. Med., in press.
2) Hodge, MacLachlan, Bloor, Welch, Kormberg & Falkenheim, J. Biol. Chem. 1941, **139**, 897.
3) Best, Hartroft & Sellers, Gastroent., 1952, **20**, 375.
4) Ueda, Nihonrinshyo (Jap.), 1953, **11**, 863.
5) Cayer & Cornatzer, Gastroent., 1952, **20**, 385.
6) Hartroft, Ann. N. Y. Acad. Sci., 1954, **57**, 633.
7) Amano, Saishinigaku (Jap.), 1952, **9**, 230.

The Tohoku Journal of Experimental Medicine, Vol. 65, No. 4, 1957

Age-adjusted Death Rates for Leukemia and Aleukemia in 16 Countries

By

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The author have calculated the age-adjusted death rates for leukemia and aleukemia in 16 countries for the years from 1950 to 1952, by the data presented in " Epidemiological and Vital Statistics Report, Vol. 8, No. 4 " published by W.H.O. in 1955. The standard population applied in the calculation of adjustment was total population of male and female in Japan classified by age group in 1950. The results are as shown in Table I. Both in males and females, U.S.A. White, Denmark, Sweden and Norway belong to the countries of high rates and Japan to those of the lowest rate.

TABLE I

Age-adjusted Death Rates for Leukemia and Aleukemia in 16 Countries, 1950–1952

(Rates per 100,000 population, using as standard population that of Japan in 1950)

	Male	Female		Male	Female
Canada	4.5	3.5	Norway	5.2	4.2
U.S.A. (White)	6.0	4.4	Netherlands	4.7	3.7
U.S.A. (Nonwhite)	3.7	2.7	England and Wales	4.1	3.1
Japan	2.0	1.5	Scotland	3.9	3.0
Germany, Fed. Rep.	4.1	2.9	Sweden	5.5	4.1
Denmark	5.8	4.2	Switzerland	5.0	3.7
Finland	3.4	3.0	Australia	4.6	3.5
France	4.0	2.9	New Zealand	5.1	3.3
Italy	3.8	2.8			

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Experimental and Clinical Observation on Acute Rising Intracranial Pressure

The First Report of Studies about Pressure in Cranium

By

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(Received for publication, July 31, 1956)

Since Naunnyn[1] stated in 1881 that increased pressure in cranium (P.I.C.) resulted with the rising of blood pressure by stimulating dura mater, and Cushing[2),3)] reported about the relation between rising P.I.C. and the general status in the earlier part of this century (1901), many studies about P.I.C. have been done by various investigators and have contributed to the study of neurosurgery and neurophysiology. The rising of blood pressure, bradycardia, and the increase of pressure pulse which are believed to be the result of rising P.I.C. is well known as Cushing's phenomena, and these signs are written as a sign of increased P.I.C. in many text books. Recently, however, Evans[4),5)] and others mistrusted this phenomena, saying that they did not observe it in their clinical experiments.

The object of this study is to know acute rise of P.I.C. has influence upon brain, heart, respiratory functions and general status. The influeences upon EEG, ECG, respiration and general status in relative to acute rise on pressure in ventricular system have been recorded.

Experimental

Method and Materials

A) Animal experiment

Unanesthetized adult cat weighing 2.5–3 kg. was strapped to a frame on prone position. In order to prevent even a slight motion during the course of the experiment the head was tightly fixed with Horsley-Clark's instrument. After local anesthesia, trepanation was made in front of the coronal sutures in order to bore a small hole. EEG was observed by bipolar leading of cortical and subcortical activities through the small hole. The 2nd lead of ECG was adopted. For the respiration, the

alteration of electrical resistance of " Sulphate of Zinc " in a rubber tube wrapped around the chest was recorded simultaneously with above elements on the inkwriting EEG machine during the experiment. P.I.C. was observed as the pressure in the cisterna magna. The recording of P.I.C. was made by inserting a long tube (1.5 mm. in diameter) into the cisterna magna vertically. Following administration of 0.9% saline solution which was warmed to 37°C and slowly injected into the ventricles at the rate of 100 mm. H_2O per 30 seconds, EEG, ECG, respiration and general stutus were observed.

B) Clinical experiment

In seven cases of epileptics, EEG (by Berger's leading), ECG (the 2nd lead) and respiration were recorded simultaneously on the inkwriting EEG machine throughout the experiment. P.I.C. was measured by inserting a rubber catheter into the lateral ventricle, which was connected to a manometer. Following injection of 0.9% saline solution which was warmed to 37°C intraventricularly through the ventricular rubber catherter at the same rate as in Experiment (A) up to 1,000 mm. H_2O, EEG, ECG, respiration and general status were observed.

Results

A) Animal experiment (Table I)

On EEG of cats, α wave activities decreased, δ wave activities increased, the base line moved and then the amplitude of EEG gradually reduced accompanied with rise of P.I.C. EEG became quite flat when P.I.C. reached 1,500 mm.H_2O–2,000 mm.H_2O, and this tendency of the cortex was greater than that of the subcortex. ECG showed bradycardia with rise of P.I.C., and then ventricular extrasystole, alternate pulse and atrioventricular block started to appear from about 500 mm.H_2O in several cases. Respiration became irregular and bradypnea appeared gradually, and when P.I.C. was over 1,000 mm.H_2O, respiration had a tendency to become superficial and it eventually stopped entirely (Fig. 1)

B) Clinical experiment

On EEG of seven epileptics, α wave activities decreased, δ wave activities increased, movement of the base line became greater accompanied with rise of P.I.C. and the amplitude had a tendency of decrease. However, great difference was found in each case; for instance, there were cases with changes like case 4 (Fig. 2), while there were cases without changes like case 3 (Fig. 3). On ECG, the same tendency of changes was found in some cases i.e., ventricular extrasystole, sinus arrhythmia, or atrioventricular block as in animal experiment while no pathological discharge in other cases. In some cases pulse rate increased, but it may be due to psychic anxiety caused by headache or the like. In those with

TABLE I

Changes of ECG, Pulse and Respiration when P.I.C. Increased until EEG was Flat in Cat

No. of cat	Quantity of injected saline solution	Initial P.I.C mm H_2O	P.I.C. when EEG is flat	Change of pulse rate per min.	Change of respiration rate per min.	Abnormal discharge of EEG
11	60	20	1.500	180→186	36→18	V.E.S.
13	50	20	1.400	114→120	36→18	V.E.S. S.A.
14	60	40	1.400	186→182	42→18	V.E.S. S.A.
16	60	60	2.000	198→ 98	24→15	V.E.S. S.A.
17	45	30	1.500	150→ 90	30→15	V.E.S. S.A.*

* V.E.S. : Ventricular extrasystole. S. A. : Sinus arrhythmia.

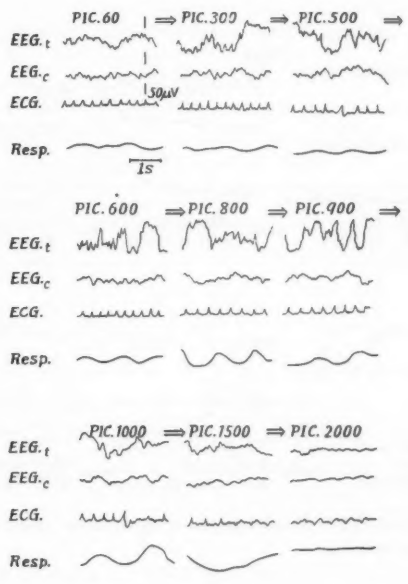

Fig. 1. Changes of EEG (cortex, subcortex), ECG and respiration which accompanied with rise of P.I.C. in cat. EEG_T : EEG of subcortex. EEG_C : EEG of cortex. P.I.C. : pressure in cranium, mm. H_2O.

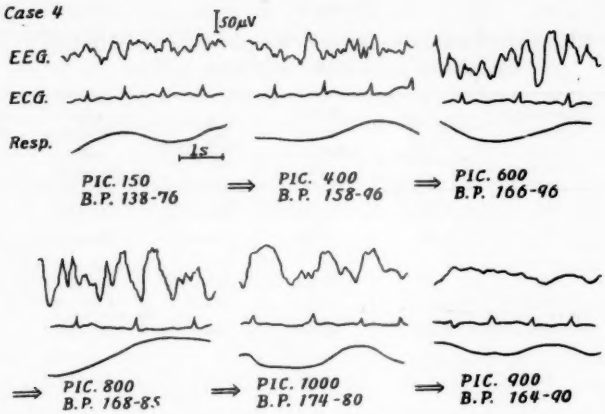

Fig. 2. (see text). P.I.C.: pressure in cranium, mm H_2O. B. P.: blood pressure, mm Hg.

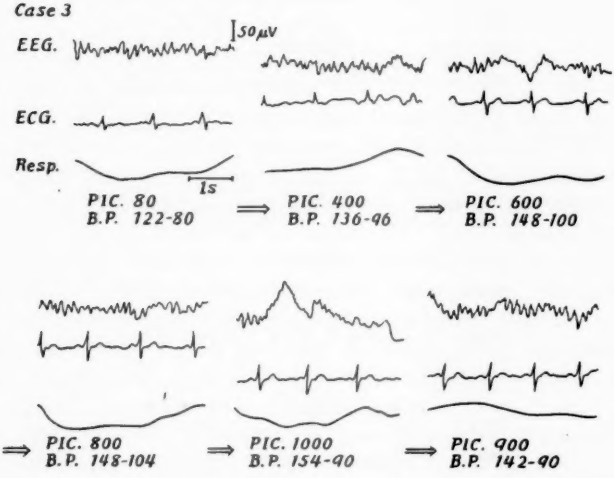

Fig. 3. (see text). P.I.C.: pressure in cranium, mm H_2O. B. P.: blood pressure, mm Hg.

few complaints, pulse rate decreased as in the animal experiment. Generally, though patients did not have any complaints up to about 500 mm.-H_2O of P.I.C., they began to complain of headache, nausea, vomiting and yawning by further increase of P.I.C. Even in such case, however, different reaction was noticed between each other. Some patients did not

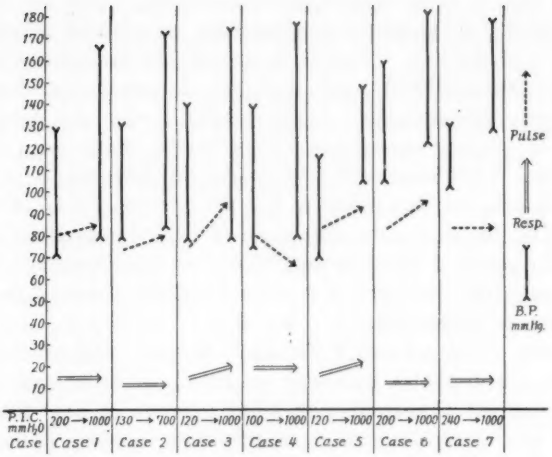

Fig. 4. Changes of Pulse, respiration, blood pressure and pulse pressure when P.I.C. increased to about 1,000 mm H_2O, from initial pressure in man.

TABLE II

Changes of EEG, ECG and General Status when P.I.C. Increased to about 1,000 mm. H_2O from Initial Pressure in Man

	Case 1 29 year old	Case 2 28 year old	Case 3 24 year old	Case 4 17 year old	Case 5 27 year old	Case 6 25 year old	Case 34 year old
Changeableness of EEG	##	##	+	##	##	+	+
Abnormal Discharge of EGG	S.A.* at 1000 mm H_2O	V.E.S.* from 300 mm H_2O	–	V.E.S. S.A. from 400 mm H_2O	S.A. from 600 mm H_2O	–	S.A. from 100 mm H_2O
General Status	Headache from 700 mm H_2O	Headache from 600 mm H_2O	Headache Nausea from 600 mm H_2O	No complaint	Headache Yawning from 600 mm H_2O	Nausea from 600 mm H_2O	No complaint

* V.E.S. : Ventricular Extrasystole. S. A. : Sinus Arrhythmia.

suffer from pain at all even at 1,000 mm.H_2O of P.I.C. The blood pressure rose and the pulse pressure increased simultaneously with the rise of P.I.C. (Fig. 4 & TABLE II).

COMMENT

From the above experiments, when rise of P.I.C. occurs in the ven-

tricles as in case of acute ventricular hemorrhage, brain can maintain its function until P.I.C. increases considerably, in spite of a comparatively large quantity of bleeding. That is, change of EEG in cats become manifest from about 1,000 mm.H_2O, and amplitude do not become flat till P.I.C. reaches about 1,500 mm.H_2O–2,000 mm.H_2O. On the other hand, in some cases of clinical experiment, EEG hardly shows any change until P.I.C. reaches 1,000 mm.H_2O. Whatever it may be, it is minimum. Therefore, it may be impossible to tell the present P.I.C. of the patient from his EEG, because wide difference of EEG is observed individually with the increase of P.I.C., though EEG has fixed tendency of decrease of α wave activities, increase of δ wave activities, move of the base line, and reduction of amplitude.

According to Motokawa,[6] Zeniya,[7] Berger[8] and others, change of EEG which accompanies with rise of P.I.C. is little, and MacCarty[9] says most common abnormality of EEG is move of base line, and it is more likely to be seen in young patients than in old. Pathological discharges of ECG, i.e., ventricular extrasystole, alternate pulse, atrioventricular block, etc. appear from about 500 mm.H_2O in cats, and from about 400 mm.H_2O in clinical cases. These changes may be caused by the unbalance of both functions of the vagus and the sympathetic nerve as a result of anemia in the center of the autonomic nervous system which accompanies with rise of P.I.C. Pulse rate, respiration and blood pressure change with acute rise of P.I.C. as Cushing stated; the tendency of bradycardia, bradypnea, rise of blood pressure and increase of pulse pressure are seen in this experiment. Probably, owing to headache and psychiatric anxiety, all patients don't always show bradycardia and bradypnea. Observation of rise and fall of P.I.C. within a few days after the operation is noticed, as has been reported in the 2nd communication.[10] In those cases who show comparatively chronic rise of P.I.C., these changes are not always in accord with Cushing phenomena; the tendency of Cushing phenomena is seen only when P.I.C. reaches about 1,000 mm.H_2O, and condition of pulse rate, respiration and blood pressure differ with patients. Therefore, it may be impossible to know the degree of P.I.C. from these conditions. The conditions of pulse rate, respiration and blood pressure show different attitude in case of acute rise of P.I.C. and chronic rise of P.I.C., in the former there are some examples that patients do not suffer in general status so much until P.I.C. reach 1,000 mm.H_2O, but in the latter there are many patients who fall into indistinct consciousness, complains of hallucination, anxiety and urine incontinentia when P.I.C. reaches 1,000 mm.H_2O.

Summary

EEG, ECG, respiration, general status and so forth which accompany with rise of pressure in cranium, especially, in ventricles are reported.

1. When pressure in ventricles rise acutely, it may be without danger even at high degrees. On seven epileptics, some of them do not complain of pain so much, even P.I.C. rises to 1,000 mm.H_2O, which is usually dangerous for patients with brain tumor.

2. In both animal and clinical experiments, pulse rate, respiration and blood pressure change in accordance with Cushing phenomena; bradycardia, bradypnea and rise of blood pressure accompany with P.I.C. However, emphasis is made on the point that Cushing phenomena can not be applied to every case. Those with chronic rise of P.I.C. which are observed after brain operation do not accord with it.

3. EEG shows a fixed tendency with rise on P.I.C.; that is, α wave activities for a time at the beginning of the course of the experiment, but they decrease gradually, and δ wave activities increase, the base line of EEG looses its balance and the amplitude reduces with rise on P.I.C. In cats, EEG almost diminishes at 1,500 mm.H_2O. In clinical experiments the above mentioned tendency is noticed when P.I.C. rises to 1,000 mm.H_2O. EEG activities, differes by individuals but generally the changes are little, and few are without change.

4. Some pathological discharge of ECG, i.e., ventricular extrasystole, sinus arrhythmia, alternative pulse, atrioventricular block, etc., accompany with rise of P.I.C. Probably, these tendencies are caused by unbalance of both the function of vagus and sympathetic nerve, as the result of anemia in center of autonomic nervous system, which accompanies with rise of P.I.C.

References

1) Naunnyn & Schreiber, Arch. exper. Path. Pharmak., 1881, **14**, 1.
2) Evans, Arch. Surg., 1951, **63**, 107.
3) Cushing, Johns Hopkins Hosp. Bull., 1901 **12**, 290.
4) Cushing, Amer. J. Med. Sc., 1903, **125**, 1017.
5) Browder & Mayer, Arch. Surg., 1938, **36**, 1.
6) Zeniya, Tohoku Igaku Zasshi, 1951, **46**, 128.
7) Motokawa, Noha (EEG) (Jap.), Nanjoshoten, Tokyo, 1947, p. 319.
8) Berger, Arch. Psychiat., 1931, **94**, 16.
9) MacCarty, Electroencephalogr., 1953, **5**, 203.
10) Suzuki, Notoshinkei (Jap.), 1956, in press.

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Change of Pressure in Cranium following Various Operations on the Brain*

The Second Report of Studies about Pressure in Cranium

By

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We need not emphasise that the change in the pressure in cranium (abbreviated as PIC hereunder) following a neurosurgical operation has a close bearing on the after-treatment and the prognosis of the patient, but we have the impression that in the past merely symptomatic measures have been resorted to, based upon scanty inferences on the PIC drawn with the general conditions, the pulse, the breathing, the blood pressure, the temperature etc. of the patient, as clueses.

In our Surgical Clinic under Prof. Katsura, however, we have for some years obtained excellent results by applying timely and most appropriate postoperative measures indicated by the measured PIC. In the meanwhile, we could observe some interesting findings in the post-operative change of PIC specific to various surgical brain diseases.

And our observations on PIC to reduce intracranial pressure with hypertonic glucose solution applied intravenously and relationship of PIC and headache, consciousness will be described.

Experimental

Method and Materials

As previously reported[1),2)] in my paper, we have been using an easily manipulated device for measuring the PIC, attached to a polyethylene tube which can be inserted into the lateral ventricle of one side through the forehead or occiput.

5 cases of corticotomized epilepsia genuina, 20 cases of benign supratentorial tumors, 13 cases of benign subtentorial tumors, 2 cases of malign supratentorial tumors, 3 cases of malign subtentorial tumors, 1

* A part was read before the 13th General Meeing of the Japanese Society of Neurosurgery, Tokyo, Oct. 25, 1954.

case of brain abscess, 4 cases of metastatic brain tumors, 6 cases of hypophyseal tumors and 4 cases of brain injuries etc., or 58 cases in total (excluding pneumoventriculography) of which we will hereunder report on the results obtained in representative cases, for contributing something to serve as guide in the future.

Results

1) Change of PIC following corticoectomy in cases of genuine epilepsy

The change of PIC following excision of unilateral area 6aβ of casey, of genuine epilepsy, a disease that can be said to accompany scarcely any organic degeneration of the brain, was as shown in Fig. 1. In such cases the PIC rose to the maximum in 30 to 40 hours after the operation, and at the highest, the value usually remained below 300 mm.H_2O. Thereafter, the PIC subsided gradually, recovering the normal level in 70 hours or so. Thus, in a moderate surgical invasion as in the above cases, we have not to make much of a heightening of PIC due to brain edema, and a dehydrating measure by means of intravenous administration of hypertonic glucose solution in such a case is little effective in lowering the PIC but in some cases it positively though slightly raises the pressure. (Fig. 1)

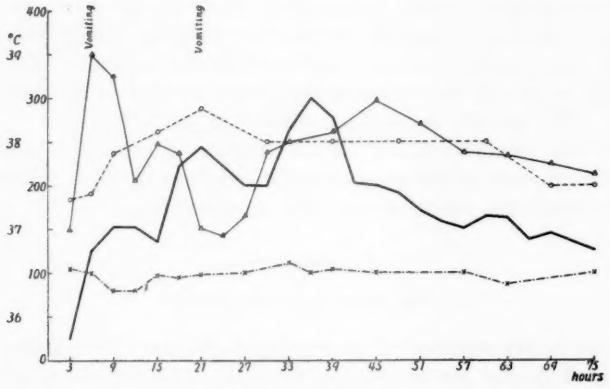

Fig. 1. X. Yamada. Epilepsy. Corticoectomy of right 6aβ. (see text). ——— PIC, —— Temperature, - - - - Respiration, - - - - - - Pulse, >——< Blood pressure.

The explanation of the lettering and the expressions given above is common to all the Figure 1, 2, 3, 4, 5,6, 7, 8, 9, and 10.

2) Change of PIC following extirpation of benign supratentorial tumors

Such tumors comprise locally circumscribed ones as parasagittal meningioma and unlocalised ones as benign glioma, and the behavior of the PIC subsequent to operations for excising such tumors showed difference by their kinds.

When unlocalized glioma was operated upon, the PIC ran up to 500 mm.H_2O in a short time after the operation in some cases and in the other, rose to the same level but slowly within some 30 hours, but at any rate, the maximum rarely exceeded 600 mm.H_2O. The value rose to the highest in 30 to 60 hours after the operation and began to subside from around the 70th hour to the level of nearly 350 mm.H_2O. (Case 1)

A tumor of this kind tending to be diffusive are hardly extirpated wholly by operation, and it is undeniable that the cerebral edema is severe in such a case. High concentration glucose solution injected intravenously is considerably effective in reducing the PIC, which temporarily falls by 200 to 300 mm.H_2O, but in an hour after the injection, it rerises to the pre-injection value or even above it in some cases. (Fig. 11) Thus, seeling that the PIC reducing action of hypertonic solution is only transient and that brain edema reappear secondarily thereafter, some[3] deny the utility of the injection, but when the PIC is constantly heightened and the blood circulation is obstructed, it may be an effective means to prolong the state of disturbed brain function for even a short time, to reduce the PIC, were it only transiently, and assure improved blood circulation in the brain. Shenkin[4] recommends intravenous injection of hypertonic glucose solution as a better method than brain puncture, for the former is effective in improving the blood circulation. A repeated injection, however, should be applied with care, as it may induce general dehydration to the detriment of the patient.

Following extirpation of localized slow-developing tumors, such as cerebral meningioma, the rise of PIC is smaller than in the preceding, being not much different from the state following corticoectomy of epileptic cases, except that the recovery of the normal PIC level comes somewhat later. (Case 2)

Case No. 1 (Fig. 2)

X. Aomatsu. Girl of 12 years old. Ependymoma in the left parietal region.[5] After the operation, the PIC gradually rose to 460 mm. H_2O in 9 hours, severe headache was complained of and the patient showed signs of uneasiness, complaining of hallucinations of small animals. 100 cc of 50% glucose solution was injected intravenously, her PIC lowered by about 200 mm. H_2O and the patient was reliefed to some extent of hallucination and headache. The PIC rerose in about one hour, but her headache remained alleviated somewhat longer. In about 50 hours after the operation, the PIC began to fall, gradually coming down to around 350 mm. H_2O and the patient left the hospital

24 days after the operation, entirely cured. When the rise of PIC is of this extent, the changes in pulsation, respiration and blood pressure remain independent of the change in PIC, and we could not confirm any sign of Cushing's phenomenon.

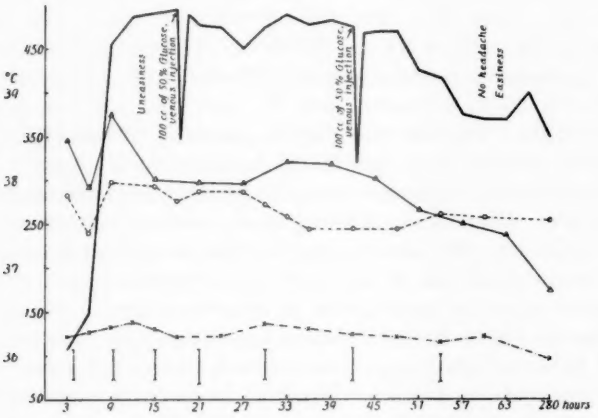

Fig. 2. Case No. 1 X. Aomatsu. Ependymoma in the left parietal region. Extirpation. (see text).

Case No. 2 (Fig. 3)

X. Ishikawa. Male. 41 years old. Meningioma in the left parietal region. The tumor was a parasagittal meningioma of the size of $8\times6\times3$ cm. It was extirpated completely, though the operation was accompanied by rather profuse bleeding.

After the operation, the PIC rose slowly, attaining 200 mm. H_2O in 20 hours and to the maximum of 300 mm. H_2O in 39 hours, but did not come down to any perceptible extent thereafter, always fluctuating between 200 and 250 mm. H_2O. His consciousness was not entirely disturbed during his cource of recovery, complaining of headache, and was discharged clinic completely curred 50 days after the operation.

3) PIC following excision of malign supratentorial tumor

When the supratentorial tumor is a malign diffusive glioma, it is difficult to extirpate it entirely in most cases, and when a part of it is left unexcised, a very serious rise of PIC follows the operation. Even repeated ventricle puncture cannot prevent a steep rise of PIC to something more than 1,000 mm.H_2O. A treatment with hypertonic glucose solution for dehydration brings only transient relief, the PIC soon rising to a level higher than before the treatment. In such case, however, this serious rise of PIC is survived and the general conditions show improvement,

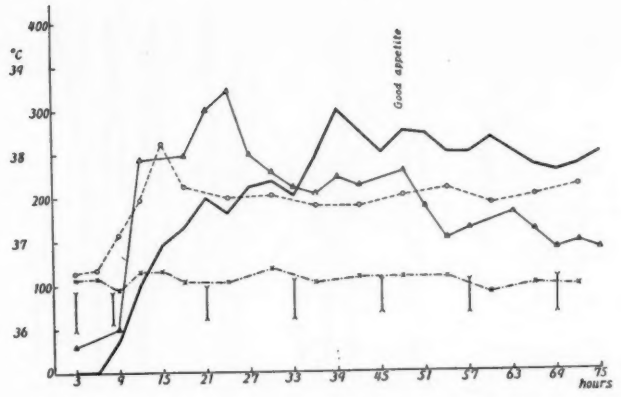

Fig. 3. Case No. 2 X. Ishikawa. Meningioma in the left parietal region. Extirpation. (see text).

the PIC begins to subside in around 80 hours after the operation and finally stabilizes between 500 and 600 mm.H_2O.

Case No. 3 (Fig. 4)

X. Sato. Male. 46 years old. Glioblastoma multiforme of the right parietal region. Upon opening the upper right temporal region a diffusely developed tumor was found and excised as wholly as possible. After the operation, his consciousness began to be turbid, vomiting became frequent and his PIC rose makedly. Attempts to control the rise of PIC by intravenous injection of 50% glucose solution proved ineffective and in 21 hours, the PIC rose to exceed 800 mm. H_2O. Then, about 8 cc of cerebrospinal fluid was extracted to depress the PIC, but presently it began to rise again. Such treatments were repeated more than a dozen times, when the patient gradually recovered consciousness and his PIC began to show stabilization at 500 mm. H_2O. Thereafter, a deep-sited irradiation with 3,000 r. of X-ray led to gradual alleviation, and the patient recovered so far as to be fully capable of walking and was discharged from our clinic in 2 months after the operation.

4) PIC following extirpation of benign subtentorial tumor

Tumors inside the temtorium always more or less obstruct the ventricular system, particularly frequently the aqueduct of Sylvius, and it may be fairly said that the result of operation on such a tumor depends on the success in removing this obstruction to open up the canal. When the success is complete, the PIC decreases and shows no perceptible rerise thereafter. Frequently after the operation a moderate rise of PIC, which forces open the aqueduct and thus drops abruptly, is followed by a sudden improvement of the patient's conditions. (Case 4) Accordingly, we may

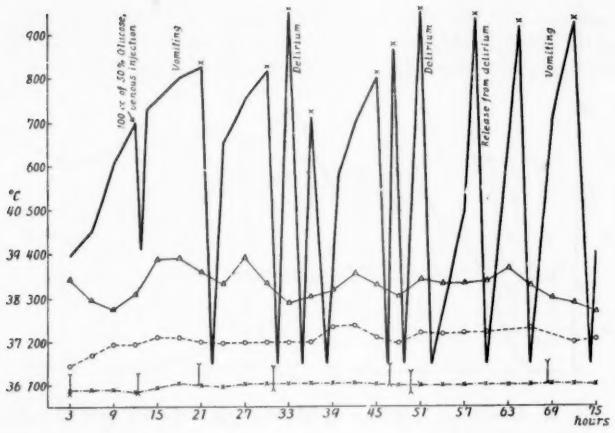

Fig. 4. Case No. 3 X. Sato. Glioblastoma multiforme of the right parietal region. Excision. (see text). × Extraction of cerebrospinal fluid.

even say that the success or failure of the operation may be evaluated from the post-operation PIC curve. On the other hand, in the case when the operation has not succeeded in eradicating the tumor and in opening up the obstructed aqueduct of Sylvius, the PIC remain high for a long time and though the progress of the disease may be held up temporarily, no positive improvment sets in, the brain edema growing worse. (Case 5) In such a case, a reoperation or a Torkildsen's operation should be considered as soon as possible. Little effect can be expected from injection of hypertonic glucose solution in a case of subtentorial tumor, the PIC being suppressed by 100–150 mm.H_2O at the highest in 30 minutes, but rising to the original level or higher in an hour. This is seemingly due to the fact that the high PIC in such cases is not caused by edema of the cerebrum, but by disturbed circulation of the cerebrospinal fluid by the obturation of the ventricular system. (Fig. 11)

Case No. 4 (Fig. 5)

X. Sato. Male. 36 years old. Meningioma in the right cerebellum.

Upon opening his lower occiput, a walnut-size tumor rich in blood vessels was found in the right cerebellum. Though the operation was accompanied with rather heavy bleeding, the tumor was successfully eradicated. Following the operation, his PIC rose and reached 350 mm. H_2O in 21 hours, but fell abruptly thereafter, to come down to the level of 150 mm. H_2O in 27 hours after the operation. Then he forgot all headache, that was so heavily felt before, began to show good appetite, and without showing sign of rerise of the PIC, discharged from our clinic on the 43rd day, quite relieved.

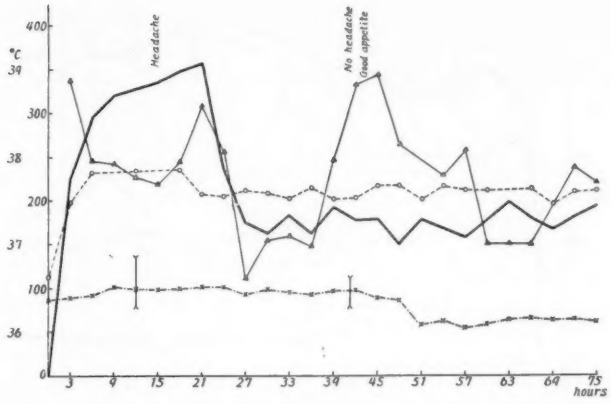

Fig. 5. Case No. 4 X. Sato. Meningioma in the right cerebellum. Extirpation. (see text).

Case No. 5 (Fig. 6)

X. Takamura. Male. 56 years old. Right acustic tumor. Upon opening the right lower occiput, a thumb-tip size tumor was found in the cerebellopontine angle, but on account of profuse bleeding, we failed in full eradication of the tumor. 6 hours after the operation, his PIC was found fluctuating near the level of 350 mm. H_2O, but showed no signs of coming down. The patient was not relieved of his headache and vomiting, his visual acuity and his hearing fell lower and lower, no improvement in his conditions was brought about, and he was discharged from the hospital about 9 months after the operation, with his both eyes totally blind and his both ears nearly deaf.

5) PIC following excision of malign subtentorial tumor

It is difficult to extirpate such a tumor infiltrating diffusely, and usually we have to leave the obstruction of the ventricular system not satisfactorily broken through, but after the operation, the PIC never was found to rise to the high as shown in the cases with malign supratentorial tumors. This is probably due to the fact that, just a slow-progressing incomplete but not a sudden and complete uretral obturation induces hydronephrose, so an abrupt and complete closure of the ventricular system acts in inhibiting the secretion by the choroid plexus and suppressing to rise of the PIC, though of course a temporary heightening of PIC must occur, as the ventricular system is obturated speedily and completely by the fast-growing malign tumor. On the other hand, it is perhaps due to the slow closure of the ventricular system that higher PIC appears more frequently in the cases with benign subtentorial tumors. As in such cases, the effect of intravenous injection of hypertonic glucose solution is not perceptibly

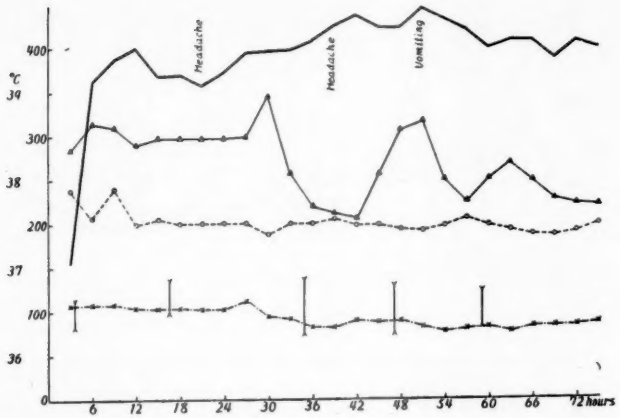

Fig. 6. Case No. 5 X. Takamura. Right acounstic tumor. Extripation unsuccessfully. (see text).

manifest in the cases with malign subtentorial tumors.

Case No. 6 (Fig. 7)

X. Saito. Boy of 7 years old. Medulloblastoma in the cerebellum. Upon opening the lower occiput, a soft tumor was found infiltrating the bilateral hemisphere of the cerebellum diffusively, covering the vermis cerebelli, and only a biopsy was taken. Thereafter, his PIC rose barely to 200 mm. H_2O, at the highest, but the patient frequently fell into fits of unconsciousness and spasms. Deep X-ray irradiation brought relief and was discharged from our clinic 50 days after the operation. He is said to be able to play harmonica at present.

6) PIC following operation on inflammatory tumor and metastatic brain tumor

In a case of inflammatory brain tumor, severe brain edema naturally follows and the PIC rises to a considerable height. Of course surgical operation in such a case should be attempted only when the acute stage has passed, a capsule has surrounded the tumor and the brain edema has abated, but since the operational process itself is followed often with considerable brain edema, a rise of PIC similar to that in the case of malign cerebral glioma is always observable. (Case 7) When a metastatic brain tumor has turned into an abscess or has diffusively invaded the brain, the PIC rises to an excessive height and shows a curve like that after operation on malign supratentorial glioma. (Case 8) When, however, no abscess is formed or the tumor is rather localized, the PIC rises only to

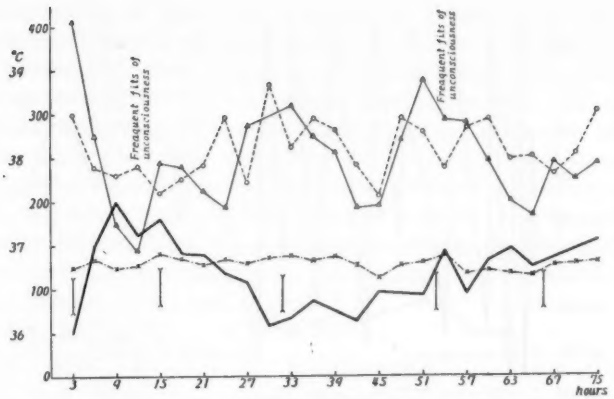

Fig. 7. Case No. 6 X. Saito. Medulloblastoma in the cerebellum. Exploratory craniotomy with biopsy of tumor. (see text).

the extent as if a benign supratentorial tumor is present.

Case No. 7 (Fig. 8)

S. Yokota. Female. 26 years old. Brani abscess. Thus, opening the right parietal region an abscess of egg-size located dorso-cranial to the Sylvius's fissure was found. It content was aspirated and a rubber drain was inserted. A culture of the pus revealed presence of streptococci. After the operation, her PIC rose rashly to exceed the 1,000 mm. H_2O mark, and removal of the cerebrospinal fluid was resorted to lower its pressure and to circumvent crises, and finally the PIC subsided to 500–600 mm. H_2O. Her consciousness returned to clarity, complaints of headache were heard no more, her appetite improved and unaided walking became posible. On the 85th day after the operation, she was discharged from our hospital.

Case No. 8 (Fig. 9)

X. Mawatari. Female. 37 years old. Metastasis of primary pulmonary cancer to cerebrum. Trepanation was made on her right forehead and the dura-mater was resected. Brain edema was found in a severe degree and by puncture about 3 cc. of pus was extracted. At the bottom of the puncture, a hard tumor closely adhering to the cerebrum was found which seemed to be diffusely infiltrating as far as to the oposite side. This tumor was excised as much as possible. After the operation, the PIC rose soon to exceed 1,000 mm. H_2O, the patient's consciousness was impaired and continued draining of cerebrospinal fluid existed for 14 days without no relief, and after 60 days of unrecovered consciousness and general marasm, she succumbed to death. Upon autopsy, a goose-egg size cancer of glandular type was found in the middle lobe of her right lung.

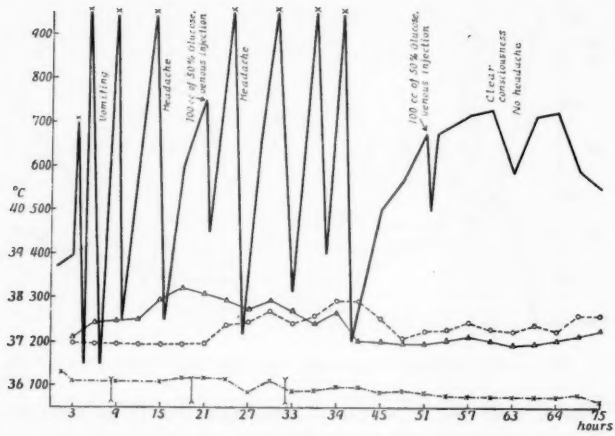

Fig. 8. Case No. 7 X. Yokota. Brain abscess. Drainage. (see text). × Extraction of cerebrospinal fluid.

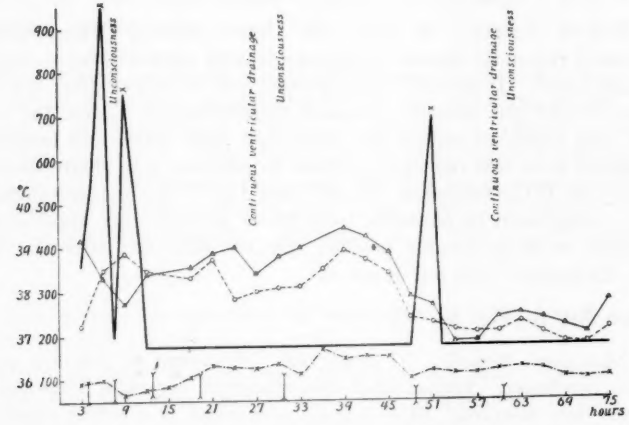

Fig. 9. Case No. 8 X. Mawatari. Metastasis of primary pulmonary cancer to cerebrum. (see text). × Extraction of cerebrospinal fluid.

7) Surgical brain diseases apt to be followed by low PIC after operation

As previously reported,[10),11)] there are cases where the PIC does not rise but lowere after the operation, causing symptoms similar to the case when the PIC rises. Such drop of PIC is frequently seen after operation for diseases of the chiasma region and for chronic subdural hematoma.

7 such cases among 58 cases, i.e., 3 among the 6 cases of diseases of the chiasma region, 2 cases of subdural hematoma, 1 case of brain injury, and 1 case of tumor in the 3rd ventricle, were encountered. It is interesting that cases of lowered PIC are often found among patients with internal hydrocephalus following pneumoventriculography. Such depression of PIC can be fairly prevented when after the operation, the spaces in the cranium are filled up with physiological saline solution and the dura mater is sutured, but when even such precautions cannot prevent subnormal PIC, we must infer that there is some disturbance in the production and absorption of the cerebrospinal fluid. The cause and treatment of such cases, have been reported previously.[9)]

Case No. 9 (Fig. 10)

X. Ejiri. Male. 46 years old. Craniopharyngioma. Lateral hemianopsia of both eyes, dilatation of the sella turcica with and calcium deposit were found, and frontal craniotomy was performed to read the pituitary region. A tumor of walnut-size, consisting of cyst filled with viscous fluid, was extirpated as much as possible, including its wall. After the operation, the PIC was so reduced as to miss even the 70 mm. H_2O level, the patient felt powerless, had headache and vomiting and his urinary output reached 4,000 cc. a day.

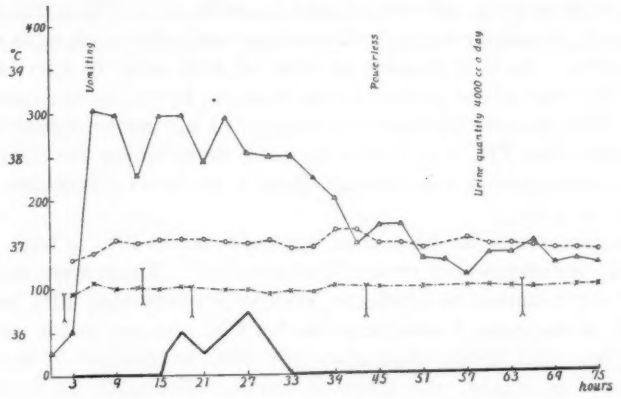

Fig. 10. Case No. 9 X. Ejiri. Craniopharyngioma. Extirpation. (see text).

Discussion and Summary

The changes in PIC, besides pulsation, respiration and body temperature, were studied several days following brain operations to treat several brain diseases as enumerated in the above, and the PIC showed changes characteristic to the diseases have been ascertained.

The so-called Cushing's phenomena,[12),13)] hitherto accepted as the best indices for proving rise of PIC, such as slow pulses, high blood pressure and increased pulse pressure, were not observable in all cases,[14)] perhaps due to the interference of many post-operative factors and the comparatively slow rise of PIC. Only when the PIC approached anything like 1,000 mm.H_2O, such effects made appearance. Thus, we have been led to deny the possibility of making inferences on the PIC from the pulsation, the respiration and the blood pressure after a brain operation.

Then, what can serve as an index of rising PIC? We must answer that there is nothing that can indicate with precision the height to which the PIC has risen. For example, take the state of consciousness of the patient. Some have illusions and lose orientation at a slight rise of PIC to 500 mm.H_2O, while others show no signs of headache and talk freely with a PIC of 1,000 mm.H_2O. So, if there is a correlation between the consciousness of the patient and his PIC, we can make no inference on the height of the latter from the state of the former. My observations have shown that, in the case where the rise of PIC is caused by obturation of the aqueduct of Sylvius or some point of the ventricular system below it, a considerable rise fails to blur the consciousness in most cases, while when a diffusive supratentorial glioma cause the brain edema to invade severely as far as to the subcortical nuclei, a mild rise of PIC to500 mm.H_2O is frequently accompanied by hallucinations and other such signs of turbid consicousness. As it is possible in some of such cases to effect improvement in the state of the patient's consciousness, by means of depressing his PIC by 50% glucose injection or removal of his cerebrospinal fluid, we may surmise that PIC is at least a factor in determining the clarity of the subject's consciousness, even though there is no direct correlation between the two.

Now, the same can be said of headache versus PIC, a high PIC not necessarily accompanying severe headache.[16),17)] There were some cases who did not complain of headache, even under very high PIC and others that had excruciating headache while his PIC was not much perceptibly overnormal. But seeing that when the PIC is reduced in the case of underarable headache, the latter is usually alleviated, an interrelation between headache and PIC cannot be denied. Generally speaking, an adrupt rise in PIC is apt to be accompanied with severe headache but there are exceptions, as reported in the previous communication,[15)] so we cannot make it a rule that acute rise in PIC is always correlated with headache. When a tumor in the vicinity of the dura mater, e.g., in the cerebral cortex, is growing rapidly, in such cases we can only predict that severe headache will develop. In such cases too, however, when the disease becomes chronic after a lapse of some time, it seems that the patient

becomes accustomed to a high PIC and pain sensation and ceases to complain much of headache. This can be affirmed when we think of the cases of brain tumors who complained of severe headache at the onset, but later on their headache had abated with time.

In conclusion, a ward on the effect of 50% glucose solution in lowering PIC is mentioned. This question was discussed by Weedund[3] in 1919 and was applied to human subject by Hadson[19] in the same year for the first time. Thereafter, the question has been in controversy.[20),21),22),23] Dandy refraining from recommending it, asserted that the PIC temporarily lower after the injection but soon rerises, and the secondary brain edema following it causes a rise of the PIC. According to personal observations, the treatment showed marked effect in some diseases, but in other cases was little effective or even raised the PIC, though slightly. It has been also ascertained that the effect lasted only about one hour at the utmost. In particular, the injection hypertonic glucose solution had little effect in the cases with no organic legion in the brain, such as genuine epilepsy and idiocy, even slightly raising the PIC in some cases, while in the cases with severe brain edema, such as malign cerebral tumor and brain abscesses, the PIC droped perceptibly, reaching the lowest level in 30 minutes, but returning to the original level in one hour. In the cases with risen PIC due to internal hydrocephalus caused by obturation of the aqueduct of Sylvius, as in subtentorial tumor, the effect of hypertonic glucose in-

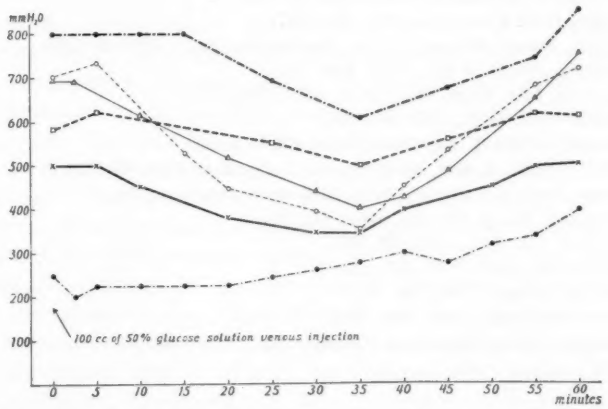

Fig. 11. Effect of intravenous injection of 50% glucose solution in lowering PIC on various neurosurgical cases. ·—·—·— Astrocytoma in the cerebellum, ———— Brain abscess, - - - - Glioblastoma multiforme in cerebrum, ------- Pinealoma, ———— Ependymoma of cerebrum, - - - - - - Epilepsy.

jection proved mediocre. Thus, the effect of hypertonic glucose solution injected intravenously in lowering PIC is only short-lived, and considering that its effect is dissimilar according to the identity of the disease, we must take care in using it in only indicated cases. (Fig. 11)

Conclusion

In this report, the author describes the results of his follow-up studies of PIC following surgical operation of representative cases of genuine epilepsy, benign and malign supratentorial tumors, benign and malign subtentorial tumors, brain abscess, metastatic brain tumor, hypophysial tumor and brain injuries undertaken at the Surgical Clinic of Prof. Katsura, Tohoku University. Besides, the problems of the correlation between PIC, and headache and the state of consciousness and of the action of hypertonic glucose solution in lowering PIC have been discussed.

References

1) Suzuki, No to Shinkei (Jap.), 1955, **7**, 280.
2) Katsura & Suzuki, Rinshogeka (Jap.), 1956, **11**, 135.
3) Dandy, J.A.M.A., 1933, **101**, 772.
4) Shenkin, J. Neurosurg., 1948, **5**, 466.
5) Suzuki, Komiyama & Toyoda, Tohoku Igaku Zasshi (Jap.), 1955, **52**, 161.
6) Torkildsen, Acta Chir. Scand., 1939, **82**, 117.
7) Fincher, Strewler & Swanson, J. Neurosurg., 1948, **5**, 213.
8) Herlin, J. Neurosurg., 1950, **7**, 403.
9) Suzuki, No to Shinkei (Jap.), 1955, **7**, 352.
10) Buffat, Helv. Chir. Acta, 1951, **18**, 315.
11) Suzuki, Kisugi, Ishibashi & Tin, Rinshogeka (Jap.), 1956, **11**, 235.
12) Cushing, Am. J. M.Sci., 1903, **125**, 1017.
13) Cushing, John Hopk. Hosp. Bull., 1901, **126**, 290.
14) Evans, Arch. Surg., 1951, **63**, 107.
15) Suzuki, Tohoku J. Exp. Med., 1956, **63**, in press.
16) Wolff, Kunkle & Ray, Arch. Neurol. & Psychiat., 1943, **49**, 323.
17) Asano, Zutsu (Headache), 1952, Igakushoin Tokyo, p. 251.
18) Weedund, Am. J. Physiol., 1919, **48**, 531.
19) Hadson, J.A.M.A., 1919, **73**, 983.
20) Northfield, Brain, 1938, **111**, 133.
21) Wilson, Surgery, 1951, **30**, 36.
22) Beel, Ann. Surg., 1953, **138**, 203.
23) Tamura, Nihon Naika Gakkai Zasshi (Jap.), 1939, **28**, 1.

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Studies on Homoimmunogen of Rabbit Kidney

Second Report

Chemical Nature of the Homoimmunogen*

By

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What kinds of lipid possess antigenicity, whether as full-antigens or as haptens, is a problem arousing interest, needless to say. Since Landsteiner[1] mentioned in 1925 that lipids can act as immunogens when mixed with a heterogenic protein, the study along this line has been repeated by many workers with lipids of known structures, cholesterol, lecithin, cephalin and cerebroside, isolated in a pure state[2)-10)], as well as with rough lipid fractions of various tissues[11),12)]. And recently Pangborn[13)] and Matalon *et al.*,[14)] on one hand, established the structure of cardiolipin which acts as a Wassermann antigen, and on the other, Masamune's[15)] and Yamakawa's[16)] schools made comprehensive study regarding the chemical nature of blood group lipids. In addition, Kobayashi,[17)] working in this Medico-chemical Institute, could separate high-molecular immunogenic lipids from human gastric mucosa and gastric cancer, which resemble in composition the lipids belonging to Subdivision *b* of class III in Masamune's classification of A, B and O group substances[18)].

As to the homoimmunogen of tissues, Sachs *et al.*[19)] injected a mixture of rabbit kidney lipids together with a pig blood serum into rabbits and discovered the antibody of the lipid mixture besides a Wassermann antibody in the immune sera. Thenceforth not a few investigators demonstrated the presence of a homoimmunogen or homoimmunogens in alcoholic extracts of various tissues (Halber and Hirszfeld[20)], Ishikawa[21)], Sakamoto[22)], Kimura[8)], Umezawa[7)], Lewis[23)], Ueno[12)], Kopeloff and Kopeloff[24)], Ota[25)]). Therefore the writer prepared a batch of alcoholic extracts of rabbit kidneys by the routine procedure to use as the material

* 25th report of Masamune and co-workers' "Chemistry and Biology of Lipids."

for the present investigation. He injected nine 5 cc. portions of a 10% rabbit kidney emulsion together with the adjuvant below in succession into 9 rabbits, and the antisera of 5 rabbits which survived were examined with respect to its complement fixation and precipitin reaction with a solution after extracting rabbit kidneys with 9 volumes of physiological saline. However, the results were all negative, indicating lack or scantiness of homoimmunogen among the water-soluble tissue components.

The material above was fractioned to separate the organ- or organ- and species-specific homoimmunogenic lipid, if present, in a homogeneous state, as will be described below. However, the final electrophoretically pure fraction proved comparatively highly but not strictly species- as well as organ-specific, as had been anticipated from the finding by Masamune *et al.*[26] on pentosenucleic acids of pig kidney and liver. Chemically examined, it was found to contain a high fatty acid or acids, glycerophosphoric acid, inositol, choline, ethanolamine and 10 or more of amino acids as its constituents but was devoid of sphingosine, plasmal and sugars, suggesting a high-molecular proteoglycerophospho-lipid.

Experiments with Comment

Isolation of the Homoimmunogen in View from Rabbit Kidneys

Material

Hashed fresh rabbit kidneys were covered under 9 volumes of alcohol and after standing at 37°C (incubator) for 1 week, the alcoholic solution was distilled to dryness. The extract was freed from the part which is insoluble in abs. alcohol at 37°C by three repetitions of extraction and evaporation. The yield was 25 g. in averages per kg. of the pulp of fresh rabbit kidneys (1 kidney weighed 7–10 g.). The procedure here is accounted for in full in the First Report.[27]

Method of Complement Fixation Assay

As indications of fractioning the material, organ- and species-specificity of the divisions were taken besides electrophoretic patterns. The specificities were examined by the complement fixation test, employing anti-rabbit kidney lipids rabbit serum no. 18 of titre 1.28×10^3 in the First Report[27]. Antisera were prepared by injecting 5 cc. of a mixture of the material above and the adjuvant of Freund (composed of 2 g. of the material, 25 cc. of a 2% egg white solution in physiological saline (in the first half period) or a 5% pig blood serum solution in physiological saline (in the last half period), 25 cc. of dry lanoline, 50 mg. of acetone-killed tubercle bacilli (human type) and 100 cc. of liquid paraffin) twice a week into rabbits. The blood was shed after 18 injections. The test was processed by progressive dilution of the antigen as follows.

0.5% alcoholic solutions of fractions of the material were added to with

9 volumes of physiological saline and the original solutions thus prepared were progressively doubled with physiological saline. 0.25 cc. were the original and diluted antigen solutions applied to the test. As the antigen control was taken 0.25 cc. of physiological saline in place of the antiserum. As the complement solution, fresh blood sera of 2–3 guinea-pigs were united and diluted that 0.1 cc. might contain 2 complement units. The antiserum was diluted so as to contain half the minimum quantity for hemolysis inhibition in 0.25 cc., which was added to the antigen solutions. As the antiserum control, 0.5 cc. of the diluted antiserum was replaced for the mixtures of the antigen and antiserum solutions. And as the hemolytic system was used 0.25 cc. of a 2.5% beef erythrocytes suspension in an immune rabbit serum, possessing hemolysin titre more than 800 and diluted to prepare a system whose 0.25 cc. contains 7 units.

0.25 cc. of the antigen solutions, original and diluted, were mixed each with 0.1 cc. of the diluted complement solution and 0.25 cc. of the diluted antiserum, and stood at 37°C for 1 hour with occasional shaking. Then the mixtures were added to further with 0.25 cc. of the hemolytic system, and after standing at 37°C for $1^1/_4$ hours, examined regarding the occurrence of hemolysis.

Absorption of the antiserum with the alcoholic extract of tissues was effected by emulsifying 5 mg. of one of the latter in 1 cc. of the former and following standing at 37°C for 2 hours with occasional shaking and in an ice-chest overnight in turn. (In a preliminary experiment 3 mg. of the extracts were found sufficient for complete absorption.) The mixtures were filtered through asbestos and the filtrates were used for the test.

Procedure of Fractionation

After several orientating experiments, a very simple procedure, which will be illustrated with an example, was devised to separate the lipid in view.

25 g. of the material were treated at first with many changes of cold dry acetone. From the solutions were recovered 11.4 g. of substance (**Fr. I**) by distillation in vacuo, and the acetone-insoluble part amounted to 10.0 g. (**Fr. II**). 1.65 g. of the latter division were then taken up in 165 cc. of dry methanol (A trace remained undissolved, which was discarded), and a 5% cadmium acetate solution in dry methanol was added in drops until no more precipitate occurred. The precipitate was washed thoroughly with dry methanol. The mother fluid and washings were combined and bubbled with H_2S. And after removing of excess H_2S by aeration, the deposit was centrifuged off and the supernatant distilled under reduced pressure. The still-residue here (**Fr. III**) weighed 0.8 g. The precipitate by cadmium acetate was suspended in methanol and decomposed with H_2S. The substance was recovered by distillation similar to above and amounted to 0.69 g. (**Fr. IV**). 0.75 g. of Fr. III was subdivided into dry ether-soluble (**Fr. V**) and -insoluble (**Fr. VI**) parts, whose yields were 0.45 and 0.27 g. respectively. The former part was electrophoretically homogeneous and fixed the complement at a dilution of 1:2560 with the antiserum no. 18.

Organ- and Species-specificity of the Antiserum No. 18 and the Lipid Fractions

1) The antiserum No. 18

Alcoholic extracts were prepared as above also from liver, spleen, brain, heart, skelet muscle, testis and erythrocyte of rabbit and kidney of human, bull, horse, pig and dog.

The antiserum No. 18 itself fixed different amounts of the complement with the extracts of the tissues above excepting rabbit spleen and dog kidney (Table I). On the other hand, after absorption with the extract of spleen,

TABLE I

Complement Fixation by the Antiserum No. 18 Together with the Alcoholic Extract of Various Tissues

− : no hemolysis, ± faint hemolysis, +~+ slight hemolysis, ++ marked hemolysis, ## complete hemolysis.

Exp.	Antiserum	Alc. extract of (in vitro antigen)	Dilution of antigens (1:)										Antiserum control
			10	2×10	4×10	8×10	1.6×10^2	3.2×10^2	6.4×10^2	1.28×10^3	2.56×10^3	5.12×10^3	
Main	No. 18	Rabbit kidney	−	−	−	−	−	−	−	−	±	##	##
		liver	−	−	−	−	−	−	−	++	##	##	
		spleen	−	+	##	##	##	##	##	##	##	##	
		brain	−	−	−	−	##	##	##	##	##	##	
		heart	−	−	−	−	−	−	±	##	##	##	
		skelet muscle	−	−	−	−	−	−	−	+	##	##	
		testis	−	−	−	−	−	++	##	##	##	##	
		erythrocytes	−	−	−	−	−	++	##	##	##	##	
		Human kidney	−	−	−	−	−	−	±	##	##	##	
		Beef	−	−	−	−	−	−	−	++	##	##	
		Horse	−	−	−	−	−	−	++	##	##	##	
		Pig	−	−	−	±	++	##	##	##	##	##	
		Dog	−	−	+	++	##	##	##	##	##	##	
Control	Physiol. saline	Rabbit kidney	++	##	##	##	##	##	##	##	##	##	
		liver	−	+	##	##	##	##	##	##	##	##	
		spleen	++	##	##	##	##	##	##	##	##	##	
		brain	++	##	##	##	##	##	##	##	##	##	
		heart	##	##	##	##	##	##	##	##	##	##	
		skelet muscle	##	##	##	##	##	##	##	##	##	##	
		testis	++	##	##	##	##	##	##	##	##	##	
		erythrocytes	##	##	##	##	##	##	##	##	##	##	
		Human kidney	−	+	##	##	##	##	##	##	##	##	
		Beef	−	+	##	##	##	##	##	##	##	##	
		Horse	−	−	++	##	##	##	##	##	##	##	
		Pig	+	##	##	##	##	##	##	##	##	##	
		Dog	++	##	##	##	##	##	##	##	##	##	

brain, testis or erythrocyte of rabbit, or of dog kidney, the antiserum still showed complement fixation with the rabbit kidney extract, but did not after absorption with any of the remaining extracts (Table IIi).

2) Frs. II, III and V

The latter procedure of complement fixation test in 1) was repeated with Frs. II, III and V as antigens instead of the extract unfractioned of rabbit kidney. The results were similar to the finding above, but the antiserum absorbed with pig kidney could fix some complement with these Frs., as shown in Table IIii, IIiii and IIiv. The specificity of the homoimmunogen in problem did not become noticeably higher, nor its reactivity with the absorbed antiserum became significantly stronger with the progress of purification. It has most probably a common partial structure with the corresponding homoimmunogen of liver, heart and skelet muscle of rabbit and kidney of human, bull and horse. That the reactivity was not elevated may be explained by the unstableness of the spacial configuration of the determinant group as in the case of Suzuki's study[28] regarding Group B lipid of human liver.

3) Frs. I, IV and VI

These fractions were discarded, because they were entirely or almost devoid of the organ- as well as species-specificity mentioned in 2), which were restricted though. As an example, the result of the assay on Fr. I is shown in Table III revealing that the antiserum absorbed with the extract of the various tissues fixed together with Fr. I the complement just or nearly to the same extent as in the control in which physiological saline was replaced for the antiserum.

Properties of the Preparation (Fr. V)

Electrophoretic pattern

As shown in Fig. 1, the preparation gave a single boundary after electrophoresis at pH 8.9, contrary to the intermediate products whose patterns are omitted in the figure.

Solubility

It was insoluble in acetone, acetic ester, dioxane, but dissolved in acidified water slightly, in methanol and plain and alkalinized water pretty well, and in ethanol, ether, petrol ether, benzene, chloroform, glacial acetic acid, pyridine, glycol and glycolmonoethylether readily.

Test-tube tests

Molisch, biuret, Liebermann-Burchard and Salkowski (for sterol) and Bial negative; Hopkins-Cole (for tryptophane) slightly positive. Hydrolyzable sulfur absent, phosphorus present.

Paper partition chromatography of the constituents

In advance, the preparation was sampled without hydrolysis for ascending paper partition chromatography with a 80% phenol-chloroform mixture (7:3 by volume). The chromatogram gave no ninhydrin spots of free amino acids, that is, the preparation proved to be practically free of contaminating amino acids and other ninhydrin-positive compounds.

The chromatography of the components was carried out by the procedure of Masamune and Hakomori.[29]

i) Water-soluble components. 25 mg. of the substance were boiled with a mixture of 2 cc. of dry methanol and 2 cc. of conc. hydrochloric acid under reflux for 5 hours. When cold, the solution was exhausted with changes of petrol ether. The petrol-ethereal solutions were combined (50 cc. in total), and after thorough washing with water, distilled to dryness in vacuo. A yellow waxy substance was given. The methanol-HCl layer after shaking with petrol

TABLE II

Complement Fixation by the Antiserum No. 18, Absorbed with the Alcoholic Extract of Various Tissues, Together with the Rabbit Kidney Extract or One of its Fractions

−, ±, +, ++, +++ signify the same as in Table I.

Antiserum absorbed with the extract of	Dilution of the antigen (1:)										Antiserum control
	10	2×10	4×10	8×10	1.6×10^2	3.2×10^2	6.4×10^2	1.28×10^3	2.56×10^3	5.12×10^3	
i. The alcoholic extract of rabbit kidney used as in vitro antigen											
Unabsorbed	−	−	−	−	−	−	−	−	++	+++	+++
Rabbit kidney	−	−	−	+++	+++	+++	+++	+++	+++	+++	+++
liver	−	−	−	+++	+++	+++	+++	+++	+++	+++	+++
spleen	−	−	−	−	−	−	−	±	+++	+++	+++
brain	−	−	−	−	−	−	−	++	+++	+++	+++
heart	−	−	++	++	+++	+++	+++	+++	+++	+++	+++
skelet muscle	−	−	++	+++	+++	+++	+++	+++	+++	+++	+++
testis	−	−	−	−	−	++	+++	+++	+++	+++	+++
erythrocytes	−	−	−	−	−	−	+++	+++	+++	+++	+++
Human kidney	−	−	+	+++	+++	+++	+++	+++	+++	+++	+++
Bull	−	−	−	++	+++	+++	+++	+++	+++	+++	+++
Horse	−	−	−	+++	+++	+++	+++	+++	+++	+++	+++
Pig	−	−	−	−	++	+++	+++	+++	+++	+++	+++
Dog	−	−	−	−	−	+++	+++	+++	+++	+++	+++
Physiol. saline (Antigen control)	−	−	−	+++	+++	+++	+++	+++	+++	+++	
ii. Fr. II of the extract of rabbit kidney used as in vitro antigen											
Unabsorbed	±	−	−	−	−	−	−	−	+	+++	+++
Rabbit kidney	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
liver	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
spleen	−	−	−	−	−	−	+	++	+++	+++	+++
brain	−	−	−	−	−	−	++	++	+++	+++	+++
heart	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
skelet muscle	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
testis	−	−	−	−	−	−	+	+++	+++	+++	+++
erythrocytes	−	−	−	−	−	−	++	+++	+++	+++	+++
Human kidney	−	−	++	+++	+++	+++	+++	+++	+++	+++	+++
Bull	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Horse	−	+	+++	+++	+++	+++	+++	+++	+++	+++	+++
Pig	−	−	−	−	++	+++	+++	+++	+++	+++	+++
Dog	−	−	−	−	−	++	+++	+++	+++	+++	+++
Physiol. saline	−	++	+++	+++	+++	+++	+++	+++	+++	+++	

TABLE II—*Concluded*

Antiserum absorbed with the extract of	Dilution of the antigen (1:)										Antiserum control
	10	2×10	4×10	8×10	1.6×10^2	3.2×10^2	6.4×10^2	1.28×10^3	2.56×10^3	5.12×10^3	
iii. Fr. III of the extract of rabbit kidney used as in vitro antigen											
Unabsorbed	±	−	−	−	−	−	−	−	−	+	##
Rabbit kidney	##	##	##	##	##	##	##	##	##	##	##
liver	##	##	##	##	##	##	##	##	##	##	##
spleen	##	##	+	−	−	±	##	##	##	##	##
brain	+	+	−	−	−	+	##	##	##	##	##
heart	##	##	##	##	##	##	##	##	##	##	##
skelet muscle	##	##	##	##	##	##	##	##	##	##	##
testis	##	##	−	−	−	±	##	##	##	##	##
erythrocytes	##	##	+	−	−	−	+	##	##	##	##
Human kidney	##	##	##	##	##	##	##	##	##	##	##
Beef	##	##	##	##	##	##	##	##	##	##	##
Horse	##	##	##	##	##	##	##	##	##	##	##
Pig	±	+	−	−	−	±	##	##	##	##	##
Dog	±	±	−	−	−	−	±	##	##	##	##
Physiol. saline	##	##	##	##	##	##	##	##	##	##	
iv. Fr. V of the extract of rabbit kidney used as in vitro antigen											
Unabsorbed	−	−	−	−	−	−	−	−	−	+	##
Rabbit kidney	+	##	##	##	##	##	##	##	##	##	##
liver	+	##	##	##	##	##	##	##	##	##	##
spleen	−	−	−	−	−	−	##	##	##	##	##
brain	−	−	−	−	−	−	##	##	##	##	##
heart	##	##	##	##	##	##	##	##	##	##	##
skelet muscle	+	##	##	##	##	##	##	##	##	##	##
testis	−	−	−	−	−	−	±	##	##	##	##
erythrocytes	−	−	−	−	−	−	−	##	##	##	##
Human kidney	+	##	##	##	##	##	##	##	##	##	##
Beef	##	##	##	##	##	##	##	##	##	##	##
Horse	##	##	##	##	##	##	##	##	##	##	##
Pig	−	−	−	−	−	−	##	##	##	##	##
Dog	−	−	−	−	−	−	+	##	##	##	##
Physiol. saline	##	##	##	##	##	##	##	##	##	##	

ether was freed from the floating tar by filtration under suction and distilled to dryness. The still-residue was heated with 4 cc. of 6 *N* HCl similar to above for 5 hours. The centrifuged supernatant of the hydrolysate was evaporated in vacuo over solid caustic soda to dryness, and the remainder was further freed from hydrochloric acid as far as possible by reevaporations with water. The final residue was taken up in 0.1 cc. of water, added to with 0.2 *N* NaOH to a pH of about 7.9 and made up to 0.5 cc. with water, followed by rejection of the insoluble part. The solution thus obtained was applied for the "first," "second" and "fourth stage" chromatography.

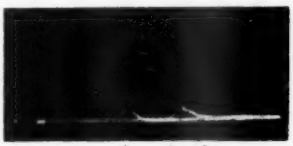
a) First stage chromatography. 0.02 cc. portions of the solution were sampled 5 cm. apart from the bottom on three 2.5×60 cm. slivers of Toyo

TABLE III

Complement Fixation by the Antiserum No. 18, Absorbed with the Alcoholic Extract of Various Tissues, Together with Fr. I of the Rabbit Kidney Extract

−, ±, +, ++, ## signify the same as in Table I.

Antiserum absorbed with the extract of	In vitro antigen diluted (1:)										Antiserum control
	10	2×10	4×10	8×10	1.6×10^2	3.2×10^2	6.4×10^2	1.28×10^3	2.56×10^3	5.12×10^3	
Unabsorbed	−	−	−	−	−	−	−	−	##	##	##
Rabbit kidney	−	−	−	++	##	##	##	##	##	##	##
liver	−	−	−	−	##	##	##	##	##	##	##
spleen	−	−	−	−	−	−	++	##	##	##	##
brain	−	−	−	−	−	+	##	##	##	##	##
heart	−	−	−	−	−	++	##	##	##	##	##
skelet muscle	−	−	−	−	±	##	##	##	##	##	##
testis	−	−	−	−	−	##	##	##	##	##	##
erythrocytes	−	−	−	−	−	++	##	##	##	##	##
Human kidney	−	−	−	±	##	##	##	##	##	##	##
Beef	−	−	−	±	++	##	##	##	##	##	##
Horse	−	−	−	++	##	##	##	##	##	##	##
Pig	−	−	−	−	+	##	##	##	##	##	##
Dog	−	−	−	−	++	##	##	##	##	##	##
Physiol. saline	−	−	−	−	##	##	##	##	##	##	



Anode

Fig. 1. Electrophoretic pattern (4/5×) of a 0.8% solution of the rabbit kidney homoimmunogen in Menzel carbonate buffer pH 8.94, I 0.11 ; current 8 mA ; temperature of test 20°C. Exposure 40 min. after starting current. The ascending limb to the left.

$$\text{Mobility}: \frac{(-1.45)\times 0.00873\times 0.3}{2400\times 0.008} = -19.8\times 10^{-5}\ \text{cm}^2\cdot\text{volt}^{-1}\cdot\text{sec.}^{-1}$$

Roshi filter paper No. 2. Ascending irrigation was effected with " Solv. II " (butanol sat. with water-phenol sat. with water-glacial acetic acid (5:2:1 by volume)), and one of the chromatograms was sprayed with lead acetate with subsequent exposure to H_2S gas. Glycerophosphoric acid was detected. A second chromatogram was treated with Dragendorf reagent and a third with iodine, and spots of choline were given. (Fig. 2 (A, B, C))

b) Second stage chromatography. 0.05 cc. of the solution was sampled for two-dimensional chromatography on a 30×30 cm. square of Toyo Roshi filter paper No. 2. Solvents: primary run, butanol-glacial acetic acid-water (4:1:2 by volume); secondary run, 80% phenol (Irrigation was effected in

Fig. 2

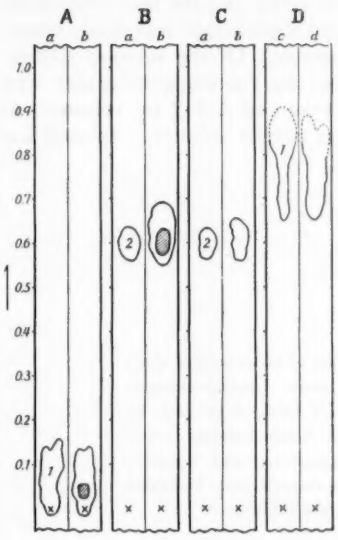


Fig. 3

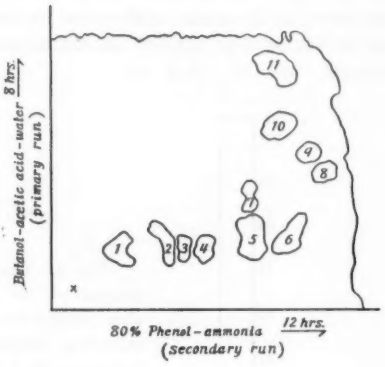


Fig. 2. First (**A**, **B**, **C**) and fourth (**D**) stage chromatograms of water-soluble components of the rabbit kidney homoimmunogen, developed by ascending irrigation with "Solv. II" and "Solv. VI" respectively. Temperature and duration of test 22°C and 24 hours respectively. Indicators: lead acetate-H_2S in **A** and **D**, Dragendorf reagent in **B** and iodine in **C**.

a, c: reference run, *b, d:* the homoimmunogen, *1:* sodium glycerophosphate, *2:* choline.

Fig. 3. Second stage chromatogram of water-soluble components of the rabbit kidney homoimmunogen. Temperature of test 25°C. Indicator ninhydrin.

1: aspartic acid, *2:* glutamic acid, *3:* serine, *4:* glycine, *5:* histidine, *6:* arginine, *7:* alanine, *8:* proline, *9:* ethanolamine, *10:* valine and/or methionine, *11:* leucine and/or isoleucine.

an ammonia-containing atmosphere). Indicator ninhydrin in butanol. As shown in Fig. 3, aspartic and glutamic acids, serine, glycine, histidine, arginine, alanine, proline, ethanolamine, valine and/or methionine, and leucine and/or isoleucine were found.

c) Fourth stage chromatography. A 2.5×60 cm. sliver was spotted with 0.02 cc. of the solution and irrigated in the ascending way with "Solv. VI" (80% phenol-glacial acetic acid-water (1:1:1)), and a spot appeared at the position corresponding to glycerophosphoric acid on treatment with lead acetate and H_2S (Fig. 2D).

In addition, inositol was detected in a hydrolysate which had been prepared by heating 25 mg. of the substance with 3 cc. of 6 *N* HCl at 100°C in a sealed

tube according to Woolley.[30] The hydrolysate was treated with petrol ether and freed from HCl as far as possible similar to above and the final evaporated residue was taken up in water. The centrifuged supernatant was again evaporated to dryness and dissolved in 0.2 cc. of water. Of the solution 0.04 cc. was sampled on a sliver 2.5×60 cm., followed by ascending irrigation with butanol-glacial acetic acid-water in the proportions of 4:1:2 by volume. As the indicator was applied an ammoniacal silver nitrate solution. Inositol was stained violet.[31] (Fig. 4)

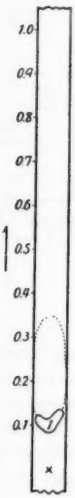


Fig. 4. Chromatogram of water-soluble components of the rabbit kidney homoimmunogen after hydrolysis with 6 *N* HCl, developed by ascending irrigation with butanol-glacial acetic acid-water (4:1:2). Temperature and duration of test 22°C and 24 hours respectively. Indicator an ammoniacal silver nitrate solution.
1: inositol.

ii) Water-insoluble components. 25 mg. of the homoimmunogen preparation were subjected to methanolysis and shaken with changes of petrol ether as in i). The methanol-HCl solution was then freed from a great part of HCl also similar to above, and the still pretty acidic residue was alkalinized against thymol blue with dilute NaOH and again shaken with ether. The ethereal solution, however, looked to have left nothing on evaporation. The petrol-ethereal solutions above were combined, and after washing with water, evaporated in the vessel, in which the ethereal solution had been evaporated, to dryness. The residue gave the strongly positive reaction in Feigl's carboxylic group test with $SOCl_2$, suggesting a fatty acid or acids. The balance of it was dissolved in 0.1 cc. of alcohol to subject to the "first stage" chromatography:—

Two 2.5×60 cm. slivers were spotted each with 0.04 cc. of the alcoholic solution and chromatograms were developed with "Solv. III" (butanol sat. with water-phenol sat. with water-ethanol (5:2:1.5 by volume)) by ascending irrigation. One of the chromatograms gave no ninhydrin spot corresponding to sphingosine. By Masamune and Hakomori's test-tube method,[32] however, the preparation was found to contain sphingosine in trace. Sphingosine must

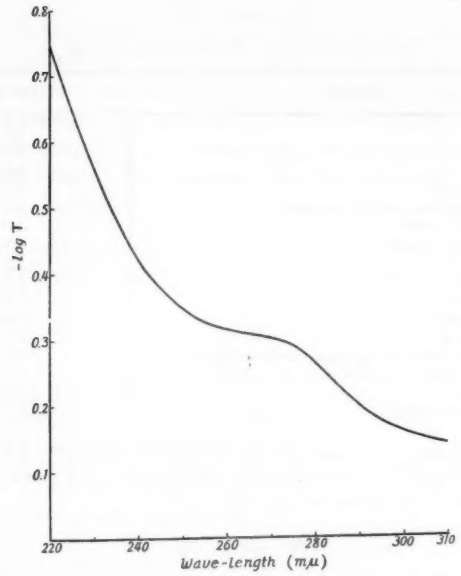


Fig. 5. Absorption spectrum of a 10 mg% solution of the rabbit kidney homoimmunogen in Menzel carbonate buffer pH 8.94, I 0.11.

have been a contamination. The other chromatogram was sprayed with Schiff reagent for plasmal with negative result.

Ultraviolet spectrum

The substance gave the spectrum in Fig. 5, indicating that it was not contaminated at all with nucleic acid which contains phosphorus, because no absorption was shown at the wave-length of 260 mμ. The slight absorption at about 275 mμ is ascribed to tryptophane (S. the section regarding test-tube tests).

Quantitative composition

The analytical figures given are embodied in Table IV.

Group potency

A 1% solution of the substance in physiological saline was prepared and 0.25 cc. portions of it and the progressively diluted solutions were added to with an equal volume of an α, a β, an anti-OS-saliva chicken- (anti-OS), a normal anti-O goat- (Goat) or an eel- (Anti-E) serum diluted to titre 16. The mixtures were stood at 37°C for 2 hours and at room temperature for 1 hour with occasional shaking, followed by placing in an ice-chest overnight. 2 drops of them were placed on a glass plate, and after addition of 1 drop of a 2% suspension of A, B or O erythrocytes, stood for 20 minutes to judge the occurrence of agglutination. As shown in Table V, no group potency was recognized, which might be a matter of course because of the deficiency of sugars as con-

TABLE IV

Analyses of the Homoimmunogen

Analysis	In per cent
N*	3.4
P†	3.2
Ash‡	1.8
Galactose§	0.0
Hexosamines as glucosamine//	0.0
Aspartic acid¶	3.5
Glutamic acid¶	4.1
Glycine¶	2.1
Serine¶	2.0
Alanine¶	0.9
Histidine¶	0.9
Arginine¶	0.8
Leucine and/or isoleucine as leucine¶	0.8
Valine and/or methionine as valine¶	0.5
Proline¶	3.0
Ethanolamine¶	0.9

* Micro Kjeldahl, † A modification of Plimmer method,[33] ‡ Pregl method without use of H_2SO_4, § Masamune and Sakamoto,[34] // A minor modification of Blix method,[35] ¶ Masamune *et al.*[36]

TABLE V

Group Potency of the Homoimmunogen

± faint hemagglutination, + slight hemaggl., ++ marked hemaggl., ## complete hemaggl.

Group of red cells	Antiserum	Dilution of the substance (1:)										Antiserum control
		10^2	2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3	6.4×10^3	1.28×10^4	2.56×10^4	5.12×10^4	
A	α	+	+	+	+	++	++	++	##	##	##	##
B	β	±	+	+	+	+	++	++	++	##	##	##
O	Anti-OS	+	+	+	+	+	+	+	++	++	##	##
O	Goat	+	+	+	+	+	+	+	++	++	++	++
O	Anti-E	+	+	+	+	+	+	++	++	++	++	++

stituents of the preparation.

SUMMARY

1. Rabbit kidneys were extracted with alcohol at 37°C, and after successive fractionation of the extract, a homoimmunogen was obtained in an electrophoretically homogeneous state.

2. The antigen is not strictly specific to rabbit kidney, but fairly common with those of liver, heart and skelet muscle of rabbit and also those of kidneys of human, of bull and of horse.

3. It was demonstrated to contain a high fatty acid or acids, glycerophosphoric acid, inositol, choline, ethanolamine, aspartic and glutamic acids, serine, glycine, histidine, arginine, alanine, proline, valine and/or methionine and leucine and/or isoleucine as constituents, but not sphingosine nor plasmal, suggesting a proteo-glycerophospholipid.

4. Its quantitative composition was investigated regarding N, P, amino acids and ethanolamine.

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References

1) Landsteiner, J. Exp. Med., 1925, **41**, 427.
2) Sachs & Klopstock, Bioch. Z., 1925, **159**, 491.
3) Ornstein, Wien. klin. Wschr., 1926, **39**, 78.
4) Ito, Hokuetsu Igakkai Zasshi (Jap.), 1928, **43**, 1135; 1929, **44**, 710.
5) Jermoljewa, Z. Immunit.-forsch., 1928, **57**, 102.
6) Suto, Shakai-igaku Zasshi (Jap.), 1930, 291.
7) Umezawa, *ibid.*, 1931, 431.
8) Kimura, Chiba Igakkai Zasshi (Jap.), 1933, **11**, 1112.
9) Wadsworth, Maltaner, E. & Maltaner, F., J. Immunol., 1935, **28**, 183.
10) Aoki, Okayama Igakkai Zasshi, 1938, **50**, 2015.
11) Kawai, Hokuetsu Igakkai Zasshi (Jap.), 1938, **53**, 1512.
12) Ueno, Kumamoto Igaku (Jap.), 1944, **20**, 126.
13) Pangborn, J. Biol. Chem., 1942, **143**, 248.
14) Matalon, Disc. Faraday Soc., 1949, **6**, 27.
15) Aikawa, Tohoku J. Exp. Med., 1949, **51**, 367; Hamasato, *ibid.*, 1950, **52**, 17; Matuda, *ibid.*, 1951, **53**, 333, 373; Masamune & Siozima, *ibid.*, 1951, **54**, 319; Masamune, Siozima & Masukawa, *ibid.*, 1951, **54**, 327; Siozima, *ibid.*, 1951, **54**, 333; Masamune & Hirata, *ibid.*, 1952, **55**, 233; Masamune, Machara & Hirata, *ibid.*, 1952, **55**, 241; Tokura, *ibid.*, 1952, **56**, 299, 307; Masamune, Tokura & Satoh, *ibid.*, 1952, **57**, 21; Masamune, *ibid.*, 1953, **57**, 310; Masamune, Machara, Hakomori & Hirata, *ibid.*, 1953, **58**, 216; Masamune, Ogawa & Akama, *ibid.*, 1953, **58**, 234; Tokura, *ibid.*, 1953, **59**, 63; Masamune, Machara & Hakomori, *ibid.*, 1954, **59**, 225; Masamune, Hakomori, Machara & Suzuki, *ibid.*, 1954, **59**, 231; Hirata, *ibid.*, 1954, **59**, 247; Hakomori, *ibid.*, 1954, **60**, 331; Masamune, Hakomori, Numabe, Akama & Kamiyama, *ibid.*, 1955, **61**, 283; Masamune & Akama, *ibid.*, 1955, **62**, 81; Akama, *ibid.*, 1956, **63**, 335; Suzuki, *ibid.*, 1956, **64**, 39.
16) Yamakawa & Suzuki, J. Bioch., 1952, **39**, 393; Yamakawa & Iida, Japanese J. Exp. Med., 1953, **23**, 327.
17) Kobayashi, Tohoku J. Exp. Med., 1956, **63**, 191.
18) Masamune, Sogo Igaku (Jap.), 1954, **11**, 181.
19) Sachs, Klopstock & Weil, Dtsch. med. Wschr., 1925, **51**, 589.
20) Halber & Hirszfeld, Z. Immunit.-forsch., 1926, **48**, 69.
21) Ishikawa, Zentralbl. Gynäk., 1928, **52**, 2571.
22) Sakamoto, Shakai-igaku Zasshi (Jap.), 1929, 1040.
23) Lewis, J. Immunol., 1933, **24**, 193; 1941, **41**, 397.
24) Kopeloff, L. M. & Kopeloff, N., J. Immunol., 1944, **48**, 297.

25) Ota, Nihon Hoigaku Zasshi (Jap.), 1952, **6**, 182.
26) Masamune, Tsuiki, Tokutomi & Kikuchi, Tohoku J. Exp. Med., 1954, **59**, 193.
27) Kitabatake, Tohoku Igaku Zassi (Jap.), in press.
28) Suzuki, Tohoku J. Exp. Med., 1956, **64**, 39.
29) Masamune & Hakomori, J. Bioch. (Japan), 1952, **39**, No. 5, 5. Ref. also to Masamune, Hakomori, Machara & Suzuki, Tohoku J. Exp. Med., 1954, **59**, 231.
30) Woolley, J. Biol. Chem., 1943, **147**, 581.
31) Hough, Nature, 1950, **165**, 400.
32) Masamune & Hakomori, Tohoku J. Exp. Med., 1956, **64**, 63.
33) Haneda, Sinokawa & Yasuoka, *ibid.*, 1944, **46**, 263.
34) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
35) Masamune & Yosizawa, *ibid.*, 1956, **64**, 53.
36) Masamune, H., Hakomori & Masamune, O., Tohoku J. Exp. Med., 1954, **60**, 347; Masamune & Hakomori, *ibid.*, 1956, **64**, 59.

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Biochemical Studies on Carbohydrates

CLCVIII. Spectrophotometric Determination of Glucuronic Acid in Co-presence of Galacturonic Acid

By

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In 1947, Dische informed of a specific color reaction of glucuronic acid. The writers made up the reaction into a quantitative-analytical method after careful study of the conditions for its occurrence.

The Analytical Procedure

0.2 cc. of 0.2% mannose is pipetted into a 18×180 mm. test-tube and 0.8 cc. of a 5–12.5 mg% solution of the unknown material in terms of glucuronic acid is added. The mixture is placed on ice, slowly added to along the tube wall with 4.5 cc. of H_2SO_4-H_2O mixture in the volume proportion of 6 : 1 and shaken under continuous cooling for 2–3 minutes. It is stood in water at 20–25°C for about 10 minutes and then heated in a vigorously boiling water-bath for accurately 2 minutes, immediately followed by placing in iced water for 2 minutes. Thereupon it is warmed to room temperature again, and after addition of 0.2 cc. of 2.5% thioglycolic acid in water, shaken. The mixture here is placed vertically in an incubator (60×90×60 cm.) at 22°±2°C for 50 hours to measure extinctions at the wave-lengths of 530 and 600 mμ against the blank by the aid of a spectrophotometer. The blank and control are carried out, replacing water and standard solutions, which contain various amounts of glucuronic acid, respectively for the solution of the unknown.

Calculation. The density increments $E_{530}-E_{600}$ are a linear function of the concentration of glucuronic acid within the range 5–12.5 mg%, and so the concentration of glucuronic acid in the solution analyzed is read from the standard curve plotted from the increments in the control.

Explanatory Experiments and Remarks

1) Fig. 1 shows the absorption spectra after the reaction of 10 mg% solutions of glucuronic and of galacturonic acid. The extinctions at the wave-lengths of 530 and 600 mμ for galacturonic acid nearly equal each

other. The spectra are plotted from the figures in Table I.

2) As shown in Fig. 2, E_{530} and $E_{530}-E_{600}$ values for glucuronic acid reach maximum at the 50th hour and remain constant for 20 hours, when final mixtures are stood at 20–22°C.

Fig. 1

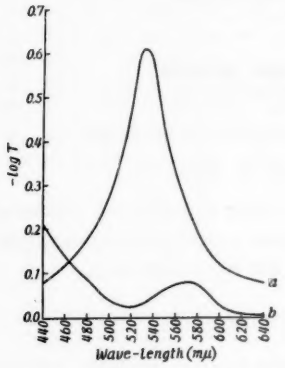

Fig. 2

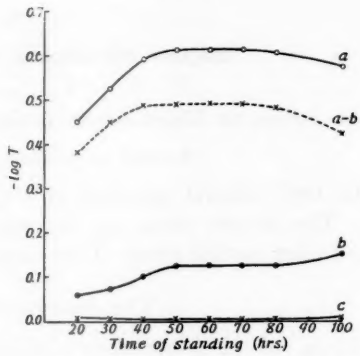

Fig. 1. Absorption spectra for 10 mg% glucuronic (*a*) and galacturonic (*b*) acids. Cell 1 cm. thick.

Fig. 2. E_{530} (*a*) and E_{600} (*b*) and the increment between them (*a*−*b*) for 10 mg% glucuronic acid and increment $E_{530}-E_{600}$ (*c*) for 10 mg% galacturonic acid as functions of time of standing the final reaction mixtures at 20–22°C.

TABLE I

Spectral Data for 10 mg% Glucuronic Acid and 10 mg% Galacturonic Acid

Wave-length mμ	−log T	
	Glucuronic acid	Galacturonic acid
440	0.075	0.210
460	0.120	0.145
480	0.175	0.085
500	0.290	0.040
520	0.464	0.030
530	0.610	0.030
540	0.575	0.050
560	0.340	0.075
580	0.195	0.075
600	0.125	0.028
620	0.100	0.005
640	0.075	0.005

3) Relation between extinctions for glucuronic acid and time of heating at 100°C of the unknown with sulfuric acid. Heating for 2 to

$2^1/_2$ minutes gives rise to the strongest absorption at the 530 mμ wave-length and the weakest at the 600 mμ wave-length. Heating for more than $2^1/_2$ minutes weakens the former absorption and strengthens the latter absorption. See Fig. 3.

4) When mixtures after addition of thionalide are stood at 20°C or a lower temperature, E_{530} and E_{600} values for glucuronic and galacturonic acids are independent upon temperature. On increasing the temperature above 20°C, E_{530} for glucuronic acid lowers gradually and the other three extinctions rise suddenly.

Fig. 3

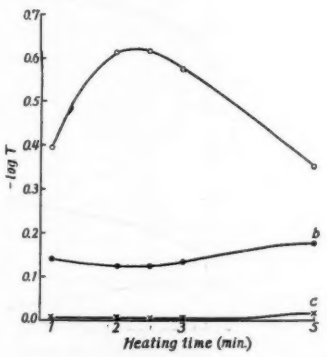

Fig. 4

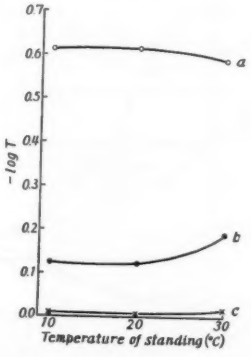

Fig. 3. E_{530} (*a*) and E_{600} (*b*) for 10 mg% glucuronic acid and $E_{530}-E_{600}$ (*c*) for 10 mg% galacturonic acid as functions of time of heating at 100°C of the hexuronic acids with sulfuric acid.

Fig. 4. E_{530} (*a*) and E_{600} (*b*) for 10 mg% glucuronic acid and $E_{530}-E_{600}$ for 10 mg% galacturonic acid (*c*) as functions of temperature of standing the reaction mixtures after addition of thionalide.

5) Concentration of the sulfuric acid reagent. Sulfuric acid (sp. gr. 1.84) was diluted with 1/5, 1/6, 1/7, 1/8, 1/10 and 1/15 the volume of water, and 0.8 cc. of the sulfuric acid solutions were mixed together with 0.2 cc. of 10 mg% glucuronic acid or 10 mg% galacturonic acid to submit to the subsequent operations in the analytical procedure. As shown in Fig. 5, E_{530} for glucuronic acid decreases swiftly with increase of the concentration of the sulfuric acid reagent, contrary to E_{600} for the same hexuronic acid which scarcely alters within the range of the proportions of H_2SO_4 to H_2O from 6 : 1 to 10 : 1. When the reagent of 6 : 1 proportion is applied, E_{600} and E_{530} for galacturonic acid show the same and lowest values. Since E_{530} for glucuronic acid varies considerably even by small change of the sulfuric acid concentration, the reagent must be prepared very carefully and preserved avoiding strictly the contact with moisture. This

is one of the main reasons why the control with standard glucuronic acid solutions has to be processed always in parallel with the main run.

6) Concentration of the thioglycolic acid reagent. Increase of the concentration of the glycolic acid reagent increases extinctions E_{530} and E_{600} for glucuronic acid almost in parallel. $E_{530}-E_{600}$ values for galacturonic acid take negative values at concentrations of the reagent higher as well as lower than 2.5%. See Fig. 6.

Fig. 5

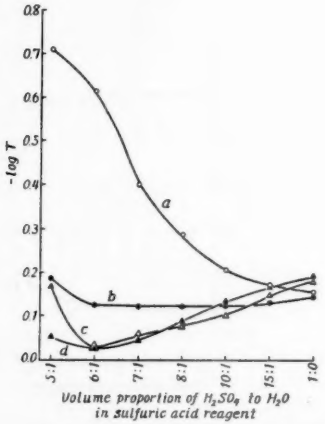

Fig. 6

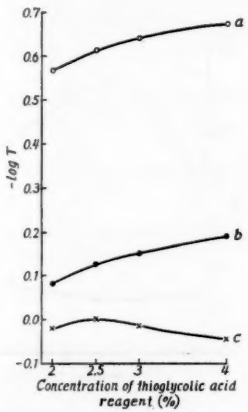

Fig. 5. E_{530} (*a*) and E_{600} (*b*) for 10 mg% glucuronic acid and E_{530} (*c*) and E_{600} (*d*) for 10 mg% galacturonic acid as functions of concentration of the sulfuric acid reagent with which the hexuronic acid solutions are heated at 100°C.

Fig. 6. E_{530} (*a*) and E_{600} (*b*) for 10 mg% glucuronic acid and $E_{530}-E_{600}$ for 10 mg% galacturonic acid (*c*) as functions of concentration of the thioglycolic acid reagent.

7) Concentration of the mannose solution. E_{530} for glucuronic acid increases abruptly and E_{600} for glucuronic acid less abruptly with the increase of the concentration of mannose, whereas increment $E_{530}-E_{600}$ for galacturonic acid is neglectable when 0.1 to 0.2% mannose solutions are applied. The concentration of the mannose reagent is another influencial factor comparable with that of the sulfuric acid reagent in the color development. Fig. 7.

8) Effect of N-acetylhexosamine upon color development of the hexuronic acids. N-Acetylglucosamine does not influence the intensity of absorption given rise to by the hexuronic acids at all in so far as its amount is twice that of glucuronic or galacturonic acid or less (Fig. 8).

9) Effect of NaCl. As shown in Fig. 9, the presence of sodium

Fig. 7

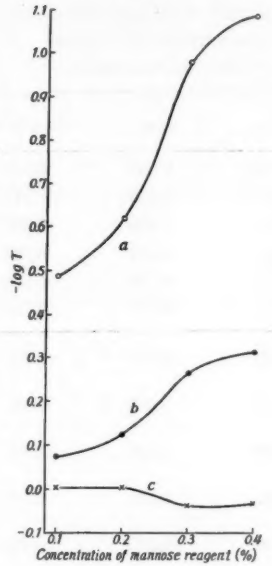

Fig. 8

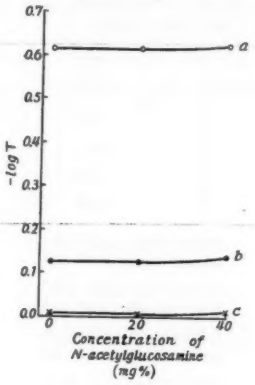

Fig. 7. E_{530} (*a*) and E_{600} (*b*) for 10 mg% glucuronic acid and $E_{530}-E_{600}$ (*c*) for 10 mg% galacturonic acid as functions of concentration of the mannose reagent.

Fig. 8. E_{530} (*a*) and E_{600} (*b*) for 10 mg% glucuronic acid and $E_{530}-E_{600}$ for 10 mg% galacturonic acid (*c*) in the co-presence of N-acetylglucosamine.

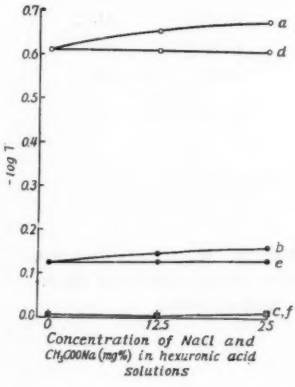

Fig. 9. Effect of sodium chloride and sodium acetate upon E_{530} and E_{600} for 10 mg% glucuronic acid and $E_{530}-E_{600}$ for 10 mg% galacturonic acid. Sodium acetate was used as an anhydrous salt. *a* and *d*: E_{530} values for glucuronic acid in the presence of sodium chloride and of sodium acetate respectively. *b* and *e*: E_{600} values for glucuronic acid in the presence of sodium chloride and of sodium acetate respectively. *c* and *f*: $E_{530}-E_{600}$ for galacturonic acid in the presence of sodium chloride and of sodium acetate respectively.

chloride raises E_{530} for glucuronic acid more than E_{600} for the same hexuronic acid, but sodium acetate is of no influence upon those absorptions.

TABLE II

E_{530} and E_{600} Values for Various Amounts of Glucuronic Acid in the Presence and Absence of Galacturonic Acid and Those for Various Amounts of Galacturonic Acid

Concentration of hexuronic acid solutions (mg%)		E_{530}	E_{600}	$E_{530}-E_{600}$
Glucuronic acid	2.5	0.130	0.030	0.100
	5.0	0.302	0.060	0.242
	7.5	0.440	0.088	0.352
	10.0	0.610	0.125	0.485
	12.5	0.754	0.150	0.600
	15.0	0.898	0.213	0.685
Mixture	Glucuronic acid 5.0, galacturonic acid 5.0	0.330	0.080	0.250
	Glucuronic acid 5.0, galacturonic acid 10.0	0.345	0.095	0.250
	Glucuronic acid 10.0, galacturonic acid 10.0	0.640	0.150	0.490
	Glucuronic acid 10.0, galacturonic acid 15.0	0.662	0.185	0.477
	Glucuronic acid 10.0, galacturonic acid 20.0	0.700	0.210	0.490
	Glucuronic acid 12.5, galacturonic acid 12.5	0.820	0.205	0.615
	Glucuronic acid 15.0, galacturonic acid 15.0	0.960	0.280	0.680
Galacturonic acid	5.0	0.010	0.010	0.0
	7.5	0.020	0.022	−0.002
	10.0	0.030	0.028	0.002
	12.5	0.050	0.050	0.0
	15.0	0.060	0.060	0.0

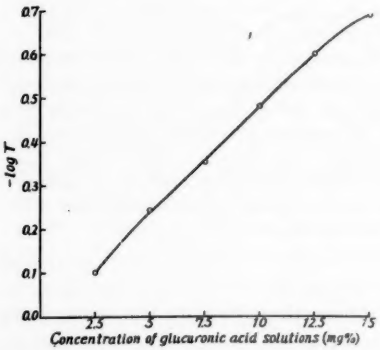

Fig. 10. $E_{530}-E_{600}$ values as a function of concentration of glucuronic acid solutions.

Both the salts are almost ineffective upon $E_{530}-E_{600}$ for galacturonic acid. In short, chloride ion is detrimental to the analysis.

10) Table II and Fig. 10 show that increments between E_{530} and E_{600} for glucuronic acid solutions follow Lambert-Beer law between the limits 5 and 12.5 mg% concentrations, whether galacturonic acid is co-present or not.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References

1) Dische, J. Biol. Chem., 1947, **171**, 725.

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Biochemical Studies on Carbohydrates

CXCIX. Spectrophotometric Determination of Galacturonic Acid in Co-presence of Glucuronic Acid

By

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(Received for publication, August 10, 1956)

Trials for spectrophotometric determination of non-amino sugars[1)-3)] by the aid of thionalide have been extended to hexuronic acids. Galacturonic and glucuronic acids both give absorption spectra with peaks at the wave-lengths of 470 and 540 mμ when submitted to the procedure below. Fortunately, however, the absorptions are markedly stronger for galacturonic acid than for glucuronic acid, and furthermore the latter hexuronic acid can be accurately estimated in the co-presence of the former hexuronic acid by Masamune and Aizawa's quantitative method[4)] of Dische reaction. Therefore, galacturonic acid in a mixture of those hexuronic acids can be calculated from the difference between the total extinction at the 470 mμ wave-length which is larger than that at the 540 mμ wave-length and the extinction at the same wave-length corresponding to glucuronic acid analyzed independently.

Technique

0.5 cc. of a solution containing 0.05 to 0.25 mg. of galacturonic acid and none or 0.05–0.4 mg. of glucuronic acid or containing a corresponding amount of a material in terms of those hexuronic acids is pipetted into a 17 × 180 mm. test tube, placed in an ice-chunk and after adding slowly 3 cc. of a H_2SO_4 (sp. gr. 1.84)-H_2O mixture (75 : 10 by volume), shaken under continuous cooling. The mixture is stood in water at 20–24°C for a few minutes at first and then in a vigorously boiling water-bath for exactly 2 minutes, followed by rapid cooling in iced water. It is removed from the iced water and stood in air. Next, 10 mg. of thionalide are dissolved in 3 cc. of the H_2SO_4-H_2O mixture above as quickly as possible by agitation with a glass rod, and 0.3 cc. of the solution at the 5th–15th minute after bringing together the thionalide and the H_2SO_4-H_2O mixture

is added to the above mixture heated, which is then stood vertically in a big incubator ($60 \times 90 \times 60$ cm.) at $22° \pm 1°C$ for 50 hours to measure extinctions at the wave-lengths of 470 and 680 mμ against the blank. The blank, in which the unknown solution is replaced with water, and the control with standard solutions containing various amounts of glucuronic acid and of galacturonic acid are carried out in parallel with the main run.

On the other hand, glucuronic acid in the unknown solution is determined according to Masamune and Aizawa.[4]

Calculation. At first, standard curves of the $E_{470}-E_{680}$ values for galacturonic acid and of those for glucuronic acid are plotted from the results in the control. The increment corresponding to the glucuronic acid in the unknown is subtracted from the total increment, and the remainder is converted into amount of galacturonic acid.

Explanatory Experiments and Remarks

1) Solutions of galacturonic and of glucuronic acid gradually get colored pink after addition of thionalide in the above procedure. Fig. 1 and Table I show the spectral data for 50 mg% solutions of those hexuronic acids after standing for 50 hours of the final mixtures. Fig. 1 shows also the spectrum for a 50 mg% solution of acetylglucosamine.

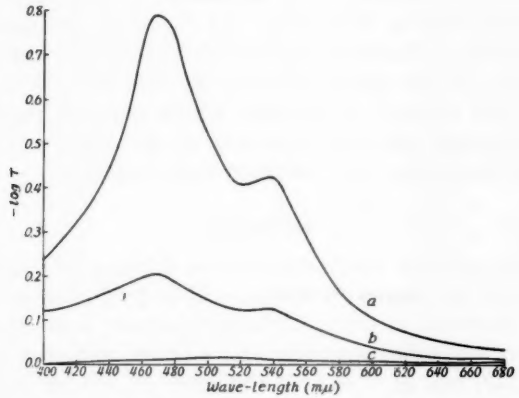

Fig. 1. Spectra for 50 mg% solutions of galacturonic (*a*) and of glucuronic (*b*) acid and of N-acetylglucosamine (*c*).

2) The reaction of galacturonic acid is almost fully developed in 1 day and that of glucuronic acid in 2 days, followed by weak continued strengthening in the both cases (Fig. 2).

3) Time of heating at 100°C. The final reaction occurs most in-

TABLE I

Extinctions for 50 mg% Galacturonic Acid and 50 mg% Glucuronic Acid as Functions of Wave-length

Wave-length mμ	Extinction	
	Galacturonic acid	Glucuronic acid
400	0.240	0.115
420	0.325	0.136
440	0.450	0.170
460	0.600	0.195
470	0.786	0.205
480	0.740	0.190
500	0.525	0.144
520	0.408	0.120
530	0.412	0.123
540	0.425	0.125
560	0.295	0.090
580	0.144	0.062
600	0.112	0.040
620	0.075	0.025
640	0.050	0.015
660	0.040	0.010
680	0.030	0.004

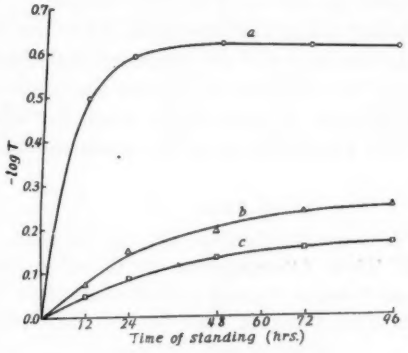

Fig. 2. Showing relationship between extinction E_{470} and time of standing the final reaction mixtures of 40 mg% galacturonic (*a*) and of 50 mg% glucuronic (*b*) acid and of 80 mg% sodium chondroitinsulfate (*c*). The specimen of chondroitinsulfate examined was prepared according to Jorpes and purified according to Masamune and Osaki.[5] It was Molisch negative and contained 33.0% galactosamine and 25.0% ash.

tensively when a solution of galacturonic acid is heated with the sulfuric acid reagent for 2 minutes, and longer heating entails depression of the reaction. In the case of glucuronic acid, however, the longer the heating is, the stronger the reaction becomes (Fig. 3).

4) Portions of a 50 mg% glucuronic acid solution were heated with

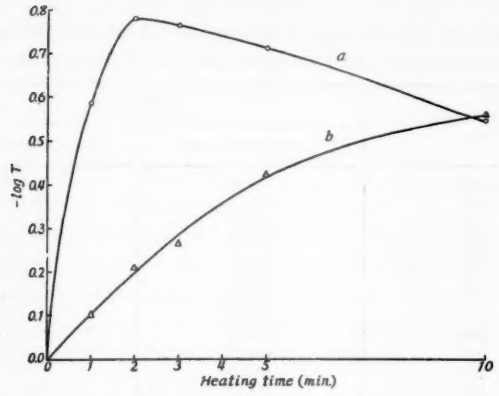

Fig. 3. Effect of time upon the extinction E_{470} of heating at 100°C of 50 mg% galacturonic (*a*) and of 50 mg% glucuronic (*b*) acid together with the H_2SO_4-H_2O (75 :10) mixture.

the sulfuric acid reagent as described in "Technique", and to the mixtures was added the thionalide solution after various durations of time from bringing together the H_2SO_4-H_2O (75 :10) mixture and thionalide. Table II shows that the longer the duration of time is, the lower the color value is. The gradient of the decrease is most gentle when the thionalide solution is applied after 5, 10 and 15 minutes from the commencement of preparation.

TABLE II

Relationship between Extinction E_{470} for 50 mg% Glucuronic Acid and the Time (Temperature 20°C) of Addition to the Hexuronic Acid Solution Heated with Surfuric Acid of the Thionalide Reagent after Bringing Together the H_2SO_4-H_2O (75 : 10) Mixture and Thionalide

Extinction	Time (min.) of addition of the thionalide reagent after bringing together the H_2SO_4-H_2O mixture and thionalide						
	1	2	5	10	15	30	120
E_{470}	0.235	0.225	0.210	0.203	0.200	0.178	0.130

5) Even if the mixture of glucuronic or galacturonic acid with sulfuric acid, heated at 100°C and cooled, is stood for several hours until it is added to with the thionalide reagent, no alteration takes place in the magnitude of extinctions.

6) Effect of NaCl on the reaction. 0.1 mg. portions of galacturonic

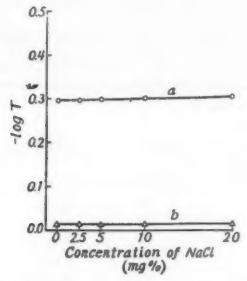

Fig. 4. Showing effect of NaCl upon extinctions E_{470} (*a*) and E_{680} (*b*) for 20 mg% galacturonic acid.

TABLE III

Effect on Extinctions for 50 mg% Glucuronic Acid of Temperature of the Incubator Wherein the Reaction Mixture Containing Thionalide Is Placed

Extinction	Temperature of the incubator (°C)		
	10～12	22	26
E_{470}	0.150	0.205	0.245
E_{540}	0.096	0.125	0.135
E_{680}	0.001	0.005	0.009

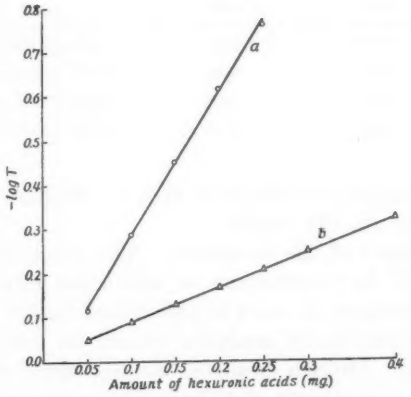

Fig. 5. Increment between extinctions E_{470} and E_{680} ($E_{470}-E_{680}$) as functions of the amount of galacturonic (*a*) and of glucuronic (*b*) acid.

acid were dissolved in 0.5 cc. of water and 2.5, 5, 10 and 20 mg% NaCl

TABLE IV

$E_{470}-E_{680}$ Values for Various Amounts of Galacturonic and Glucuronic Acids

Amount (mg.) of hexuronic acids in 0.5 cc. of their solutions		E_{470}	E_{680}	$E_{470}-E_{680}$
Galacturonic acid	Glucuronic acid			
0.05		0.130	0.008	0.122
0.1		0.297	0.015	0.282
0.15		0.473	0.023	0.450
0.2		0.640	0.025	0.615
0.25		0.786	0.030	0.756
	0.05	0.052	0.002	0.050
	0.1	0.088	0.003	0.085
	0.15	0.127	0.002	0.125
	0.2	0.171	0.003	0.168
	0.25	0.205	0.003	0.202
	0.3	0.251	0.003	0.248
	0.4	0.329	0.004	0.325

TABLE V

Extinctions at the Wave-lengths of 470 and 680 mμ for Mixtures of Galacturonic and Glucuronic Acids

Figures in parentheses are the sums of the extinctions for the hexuronic acids individually determined.

Amount (mg.) of hexuronic acids in 0.5 cc. of solutions			E_{470}	E_{680}
No. of solution	Galacturonic acid	Glucuronic acid		
1	0.08	0.04	0.260 (0.250)	0.015 (0.015)
2	0.02	0.16	0.200 (0.185)	0.007 (0.008)
3	0.12	0.08	0.440 (0.440)	0.020 (0.025)
4	0.04	0.16	0.228 (0.225)	0.003 (0.002)

solutions, and analyzed as described above. As shown in Fig. 4, NaCl is scarcely effective on the results.

7) Temperature of the incubator. The reaction occurs more and more intensively if the temperature at which the mixture after addition of the thionalide reagent is stood is higher and higher (Table III).

8) The material to be analyzed should be free from hexoses and pentoses which are Molisch positive, because these sugars give rise to absorption at the wave-length of 470 mμ like as hexuronic acids do when submitted to the present analytical procedure.

9) The increment between extinctions E_{470} and E_{680} obeys Lambert-Beer law in the range from 0.05 to 0.25 mg. of galacturonic acid and in

that from 0.05 to 0.4 mg. of glucuronic acid, as shown in Fig. 5 and Table IV.

10) The extinctions for the individual hexuronic acids are additive, when mixtures of galacturonic and glucuronic acids are dealt with. See Table V.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References

1) Masamune & Sakamoto, Tohoku J. Exp. Med., 1956, **63**, 345.
2) Masamune & Sakamoto, *ibid.*, 1956, **63**, 357.
3) Masamune & Sakamoto, *ibid.*, 1956, **63**, 363.
4) Masamune & Aizawa, *ibid.*, this volume, p. 359.
5) Masamune & Osaki, *ibid.*, 1943, **45**, 121.

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Biochemical Studies on Carbohydrates

CC. Itinsulfuric Acids in Pig Skin

First Report

By

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(Received for publication, August 10, 1956)

In 1941, Meyer and Chaffee[1] separated from pig skin a mixture of barium salts of hexuronic acid-containing mucopolysaccharides after alkaline treatment and divided it into the salts of hyaluronic acid (mucoitin) and of galactosamine-containing itinsulfuric acid by fractionated precipitation with alcohol. And they mentioned that the latter mucopolysaccharide differs in optical rotation from chondroitinsulfuric acid of beef cartilage. Later on in 1951, Meyer and Rapport[2] distinguished galactosamine-containing itinsulfuric acids from connective tissue sources into chondroitinsulfuric acid A, B and C, taking optical rotation, precipitability by alcohol and behaviour towards pneumococcal and testicular hyaluronidases as criterions. Chondroitinsulfuric acid B, as the itinsulfuric acid in skin was designated, possesses $[\alpha]_D$ value of $-50°$ and is stable to testicular hyaluronidase and its calcium salt precipitates at an alcohol concentration of 20% by volume.

More recently in 1954, Dorfman and co-workers[3] submitted a crude polysaccharide mixture from rabbit skin to electrophoresis on a slab of Hyflo Super-Cel and found that an itinsulfuric acid preparation thus purified showed the carbazol reaction for hexuronic acid corresponding only to 37% of theory on one hand, and on the other, no more than about 30% of it is degraded by testicular hyaluronidase. On these bases, the authors presumed skin to contain two sulfated polysaccharides. Meyer *et al.*[4,5] also reported that the carbazol value for uronic acid of the skin itinsulfuric acid reaches only half the calculation.

The results of the writer's experiments suggested the presence in skin of "dermoitinsulfuric acid" composed of acetylgalactosamine, galacturonic acid and sulfuric acid besides cartilage chondroitinsulfuric acid, as will be accounted for in the following.

Experiments and Comments

Fresh pig skin, shaved and freed from fatty tissue, was cut into pieces and masticated. 3 kg. of the brei given were stood in contact with 2 volumes of 0.5 *N* NaOH at room temperature (ca. 20°C) for 24 hours, and after adjusting to neutral (to pH 7.2–7.4) with 50% CH_3COOH, squeezed. The residual tissue was subjected again to extraction in a similar manner. The turbid solutions were combined and filtered through Toyo Roshi filter paper No. 2, and the filtrate was distilled in vacuo at 40°C to about 4 *l.*, added to with 3 volumes of alcohol (99%) and stood for 1 day. Most of the clear supernatant fluid was decanted out. Then the deposit was centrifuged off, washed with alcohol and ether and dried overnight in vacuo over $CaCl_2$. The dry powder weighing 30 g. was taken up in 300 cc. of water, and the insoluble part was rejected. The solution was added to with crystalline sodium acetate to 1% and adjusted to pH 6.0 with 1% acetic acid, followed by further addition of 3 cc. of saliva previously filtered with suction. The mixture was stood at 37°C for 6 hours to digest glycogen and then precipitated with 3 volumes of alcohol (99%). The deposit here was washed with alcohol and ether and dried. The dry substance (24 g.) was exhausted with decreasing volumes (7 to 5 volumes) of 90% phenol to eliminate proteins. The part remaining undissolved was washed with changes of alcohol (99%), adding 99% alcohol saturated with crystalline sodium acetate when necessary, and when it smelled of phenol no more, washed with ether, followed by drying. About 8 g. was the yield.

It was dissolved in 200 cc. of 10% NaCl, deproteinized by many repetitions of Sevag procedure employing each time 1/4 the volume of an amylalcohol-chloroform mixture (1 : 4 by volume) and dialyzed in a cellophane bag against running water for 48 hours. The filtrate was distilled under reduced pressure to about 50 cc., added to with crystalline barium acetate to 10% and with alcohol to 20% and placed in an ice-chest for 24 hours. The centrifugate was reprecipitated several times with alcohol from 10% barium acetate as above, and after washing with alcohol and ether, dried. Crop, 1.2 g. This substance was biuret −, Molisch −, Goldschmiedt ##, Neuberg-Saneyoshi ##, hydrolyzable sulfur ## and phosphorus −.

It was dissolved in 100 cc. of water, repeatedly passed through a column (2×50 cm.) of Amberlite IR-120 (H-form), and after distilling in vacuo to a syrup, added to with 100 cc. of a mixture of equal volumes of alcohol (99%) and ether. The flocculent precipitate was washed with ether and dried (**Prep. I**, 560 mg.).

After another similar run, starting with 2.6 kg. of the tissue pulp,

440 mg. of **Prep. II** were obtained.

1) 30 mg. portions of the preparations were dissolved separately in 3.0 cc. of phosphate buffer pH 7.7, I 0.2 to examine electrophoretically. Patterns showing only one boundary were given (Fig. 1). The preparations, however, gave in acetate buffer pH 4.5, I 0.2 patterns with two boundaries, as reproduced in Fig. 2, proving inhomogeneous.

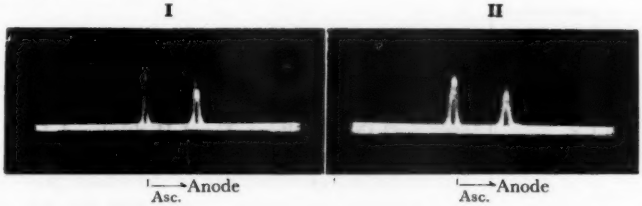

Fig. 1. Electrophoretic patterns (4/5×) of 1% solutions of Preps. I (**I**) and II (**II**) in phosphate buffer pH 7.7, I 0.2; current 12 mA; temperature 15°C. Exposure 1800 seconds after starting current. Mobility :—

Prep. I, $\frac{(-1.5)\times 0.0104\times 0.3}{0.012\times 1800}=-21.6$ cm²/volt/sec × 10^{-5}

Prep. II, $\frac{(-1.5)\times 0.0104\times 0.3}{0.012\times 1800}=-21.6$ cm²/volt/sec × 10^{-5}

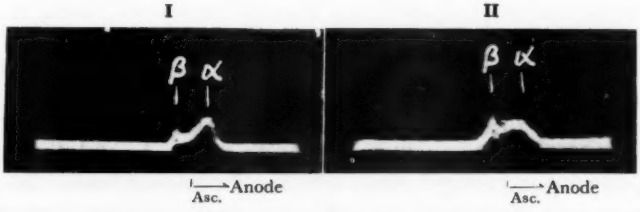

Fig. 2. Electrophoretic patterns (4/5×) of 1% solutions of Preps. I (**I**) and II (**II**) in acetate buffer pH 4.6, I 0.2; current 8 mA; temperature 17°C. Exposure 3600 seconds after starting current. Mobility :—

Prep. I, Boundary α: $\frac{(-2.75)\times 0.0097\times 0.3}{0.008\times 3600}=-27.7$ cm²/volt/sec × 10^{-5}

β: $\frac{(-2.20)\times 0.0097\times 0.3}{0.008\times 3600}=-22.2$,,

Prep. II, Boundary α: $\frac{(-2.70)\times 0.0097\times 0.3}{0.008\times 3600}=-27.2$,,

β: $\frac{(-2.20)\times 0.0097\times 0.3}{0.008\times 3600}=-22.2$,,

2) 20 mg. portions of the preparations were heated with 2 cc. of 1 *N* HCl at 100°C for 15 hours and centrifuged to remove humin. The supernatant fluid was freed from the hydrochloric acid by repeated evaporations in a vacuum desiccator containing solid caustic soda and anhydrous calcium chloride, diluting in advance with water in the second and following times (Cf. Masamune *et al.*[6]).

When a watery solution of pH 4.0 was given, it was evaporated to complete dryness and taken up in 0.05 cc. of water. 0.03 cc. portions of the solutions thus given were sampled for paper-chromatography with solvent butanol-pyridine-water (5 : 3 : 2) according to Masamune and Yosizawa.[7] In either of the preparations, spots were indicated at the positions corresponding to galactosamine and glucuronic and galacturonic acids by Partridge reagent (Fig. 3).

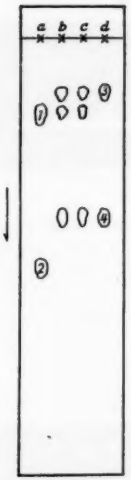

Fig. 3. Sugar paper chromatograms (2/15×) of Preps. I and II developed by descending irrigation with a mixture of butanol-pyridine-water in the volume proportions of 5 : 3 : 2. Temperature and duration of test 25°C and 60 hrs. respectively. Indicator: aniline hydrogen phthalate reagent of Partridge.

a, *d*: reference run. *b*: Prep. I. *c*: Prep. II. *1*, glucuronic acid. *2*, glucosamine. *3*, galacturonic acid. *4*, galactosamine.

The balance (510 mg.) of Prep. I was taken up in 40 cc. of water, added to with 4 g. of crystalline sodium acetate and precipitated with 5 volumes of alcohol (99%). After standing for several hours, the deposit was centrifuged off, washed with ether and dried. 640 mg. were yielded. In a similar manner, 440 mg. of the corresponding product were obtained from 390 mg. of Prep. II.

The substances given, which will be referred to as Prep. I′ and II′ respectively, analyzed as shown in Table I, confirming that they contained galacturonic acid besides glucuronic acid. Despite of similar compositions, they were possibly mixtures of glucuronic acid- and galacturonic acid-containing itinsulfuric acids. Therefore, attempts were made to fraction them into individual ingredients, and finally the following procedure was resorted to.

Of the balances of them united, 420 mg. were dissolved in 30 cc. of 1/5 *M* acetate buffer, cooled to 0°C and fractionally precipitated with alcohol. The precipitates given at alcohol concentrations of 45 to 50% (**Fr. I**), 53–60% (**Fr. II**) and 60–75% (**Fr. III**) were reprecipitated in a similar manner and dried at first in vacuo over $CaCl_2$ at room tempera-

TABLE I
Composition of Preps. I′ and II′

Analysis	In per cent		In equivalents per equivalent weight	
	Prep. I′	II′	Prep. I′	II′
Nitrogen*	2.5	2.5	0.97	0.97
Galactosamine†	33.2	33.0	1.0	1.0
Glucuronic acid‡	14.3	13.9	0.39	0.38
Galacturonic acid§	21.4	22.3	0.59	0.61
Hydrolyzable sulfur//	5.8	5.8	0.98	0.97
Acetyl//	7.9	7.6	0.99	0.96
Ash (Na_2SO_4)¶	26.1	27.3	1.98	2.08

* Micro Kjeldahl. † A minor modification of Blix method.[8] ‡ Masamune and Aizawa.[9] § Masamune, Sakamoto and Aizawa.[10] // Suzuki.[11] ¶ Pregl.

ture and afterwards over P_2O_5 at 40°C. Yielded were 125 mg. of Fr. I, 150 mg. of Fr. II and 75 mg. of Fr. III. All the fractions were Molisch- and biuret-negative but Goldschmiedt-Neuberg-positive and contained hydrolyzable sulfur but not phosphorus. And, when liberated from sodium as above, they each showed one boundary on electrophoresis in acetate buffer pH 4.5, I 0.2 (Fig. 4), and their hydrolysates gave in a paper-chromatogram only two spots at the positions corresponding to galactosamine and glucuronic acid or to galactosamine and galacturonic acid (Fig. 5). Chromatography was effected here as follows: 10 mg. portions of the fractions were hydrolyzed (1 cc. of 1 *N* HCl, 100°C, 15 hours), and after expelling off of HCl similar to above, the final evaporated residues were dissolved in 0.05 cc. of water. 0.03 cc. portions of the solutions were sampled. Analytical figures of the fractions themselves are embodied in Table II. Optical rotation in water:

$$\text{Fr. I:}\quad [\alpha]_D^{20}=\frac{-0.239\times 100}{1\times 0.87}=-27.6^\circ$$

$$\text{Fr. II:}\quad [\alpha]_D^{20}=\frac{-0.602\times 100}{1\times 0.099}=-60.9^\circ$$

$$\text{Fr. III:}\quad [\alpha]_D^{20}=\frac{-0.786\times 100}{1\times 1.20}=-65.5^\circ$$

Contrary to the finding by chromatography, the analyses disclosed that all the fractions contained both of glucuronic and galacturonic acids, which may disagree also with the result of electrophoresis. However, the mutual quantitative proportion of the hexuronic acids was different from fraction to fraction. Galactosamine was the sole hexosamine in

Fig. 4

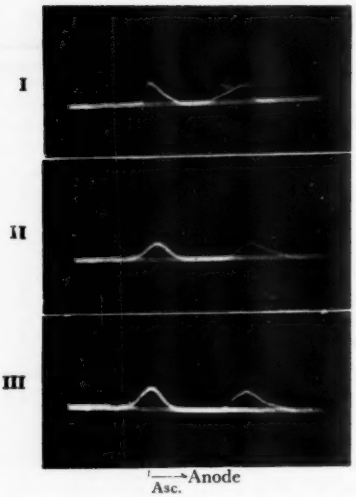

Fig. 5

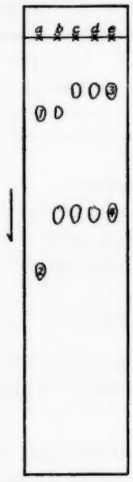

Fig. 4. Electrophoretic patterns (4/5×) of 0.8% solutions of Fr. I (**I**), II (**II**) and III (**III**) in acetate buffer pH 4.6, I 0.2; current 7 mA, temperature 17°C. Exposure 1800 seconds after starting current. Mobility :—

$$\text{Fr. I, } \frac{(-1.10)\times 0.0095\times 0.3}{0.007\times 1800} = -24.8 \text{ cm}^2/\text{volt/sec}\times 10^{-5}$$

$$\text{II, } \frac{(-1.20)\times 0.0095\times 0.3}{0.007\times 1800} = -27.1 \quad ,,$$

$$\text{III, } \frac{(-1.20)\times 0.0095\times 0.3}{0.007\times 1800} = -27.1 \quad ,,$$

Fig. 5. Sugar paper chromatogram (2/15×) of Frs. I, II and III developed with solvent butanol-pyridine-water (5:3:2 by volume). Temperature and duration of test 25°C and 60 hrs. respectively. Indicator aniline hydrogen phthalate reagent of Partridge.

a, *e* : reference run. *b* : Fr. I. *c* : Fr. II. *d* : Fr. III. *1*, glucuronic acid. *2*, glucosamine. *3*, galacturonic acid. *4*, galactosamine.

the fractions and not only the hexosamine but also hydrolyzable sulfur was present in amounts equivalent to the total sums of the hexuronic acids in respective fractions. Therefore the fractions were probably mixtures of chondroitinsulfuric acid and a new itinsulfuric acid constituted of equimolecular acetylgalactosamine, galacturonic acid and sulfuric acid. Calculated from the optical rotations and glucuronic and galacturonic acids contents of Fr. I and III, the sodium salt of the new itinsulfuric acid, which will be named " dermoitinsulfuric acid ", has an approximate specific rotation of −80°.

Further trials for isolating dermoitinsulfuric acid as well as identifying

TABLE II

Composition of Frs. I, II and III

	In per cent			In equivalents per equivalent weight		
	Fr. I	II	III	Fr. I	II	III
Nitrogen*	2.6	2.6	2.7	1.0	1.0	1.0
Galactosamine†	33.1	32.7	33.0	1.0	1.0	1.0
Glucuronic acid‡	30.8	10.7	8.8	0.86	0.30	0.25
Galacturonic acid§	4.6	22.8	25.3	0.13	0.64	0.70
Hydrolyzable sulfur//	5.6	5.5	5.6	0.95	0.94	0.96
Ash (Na_2SO_4)¶	25.0	25.8	24.3	1.90	1.99	1.85

*, †, ‡, §, //, ¶ Signify the same as in Table I.

the hexuronic acid in it will be informed of elsewhere from this Institute.

The Ministry of Education gave a grant in aid to us through the Grant Committee for Scientific Researches, which is gratefully acknowledged. H. Masamune.

References

1) Meyer & Chaffee, J. Biol. Chem., 1941, **138**, 491.
2) Meyer & Rapport, Science, 1951, **113**, 596.
3) Schiller, Mathews, Jefferson, Ludowieg & Dorfman, J. Biol. Chem., 1954, **211**, 717.
4) Meyer, Davidson, Linker & Hoffman, Science, 1954, **120**, 785.
5) Meyer, Davidson, Linker & Hoffman, Federation Proceedings, 1955, **14**, 254.
6) Masamune, Hakomori, Numabe, Akama & Kamiyama, Tohoku J. Exp. Med., 1955, **61**, 283.
7) Masamune & Yosizawa, *ibid.*, 1953, **59**, 1.
8) Masamune & Yosizawa, *ibid.*, 1956, **64**, 53.
9) Masamune & Aizawa, *ibid.*, this volume, p. 359.
10) Masamune, Sakamoto & Aizawa, *ibid.*, this volume, p. 367.
11) Suzuki, J. Bioch. (Japan), 1938, **27**, 367.

Note at proof-reading: After the manuscript was contributed to the Journal, a paper of Hoffman, Linker and Meyer appeared in Science (1956, **124**, 1252), which suggests that chondroitinsulfate B contains iduronic acid.

The Tohoku Journal of Experimental Medicine, Vol. 65, No. 4, 1957

Biochemical Studies on Carbohydrates

CCI. On the Mode of Union between Itinsulfuric Acids and Proteins in Pig Skin

By

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(Received for publication, August 10, 1956)

Meyer[1] claimed that "chondroitinsulfuric acid B" is stably linked to proteins in skin because it is separated by strong alkali from the tissue but not by neutral salts contrary to the chondroitinsulfuric acid in cartilage, which is readily extracted with 10% $CaCl_2$. Whether or not the skin itinsulfuric acids (Cf. Aizawa[2]) are firmly fixed in the tissue through normal co-valency in distinction from mucoitin, chondro- and limacoitinsulfuric acid and heparin is an important problem (Cf. Masamune[3]). As a matter of fact, the writer could not extract the itinsulfuric acids from pig skin with 10% $CaCl_2$ and the half-saturated NaCl nor even by the formalin method of Masamunė and Osaki[4] which is processed at pH 8.0 and with the half-saturated lime water (pH 9.8). However, as is dealt with in this account, 10% $CaCl_2$ releases from the skin a mixture of the itinsulfuric acids of similar composition as those obtainable by the aid of alkali[2] and that in similar yield, when the tissue is preliminarily treated with 50% urea in order to eliminate collagen present in an abundant quantity. Many years ago, Masamune[5] put forward the opinion that the linkage between a hexuronic acid-containing mucopolysaccharide and proteins in tissues is chiefly ionic and subsidiarily co-ordinate. The present result is another affirmative evidence for his view, because basic groups of collagen must be considered to take a great part in binding the itinsulfuric acids in skin.

Experimental

Fresh and shaved pig skin was freed from fatty tissue, ground in a masticator and dried in a Faust apparatus. 130 g. of the dry tissue (corresponding to 300 g. of the fresh pulp) were shaken with 7 volumes of 50% urea for 24 hours at room temperature, and percolated. The residual tissue was submitted to the same treatment over again. The filtrates

were dialyzed in cellophane bags against running water for 1 day. The inner fluids turned gelatinous. They were heated to 40°C, whereby they liquefied, and added to with 3 volumes of alcohol (99%). The deposits were united, washed with alcohol (99%) and ether and dried in a vacuum desiccator containing $CaCl_2$. The powder was freed from the part soluble in 90% phenol. The insoluble part which amounted only a little was centrifuged off, washed with alcohol (99%) and ether and dried as above. It showed neither Neuberg-Saneyoshi nor Goldschmiedt reaction.

The tissue residue exhausted with 50% urea was extracted twice with 3 volumes of 10% $CaCl_2$ for 24 hours at 20°C. The solutions were dialyzed in cellophane bags against running water for 24 hours. The occurring small precipitates were filtered off, and the filtrates united were distilled in vacuo to about 50 cc., added to with 3 volumes of alcohol (99%), and after standing overnight, centrifuged. The centrifugate was washed with alcohol (99%) and ether and dried as above. Yield 1.1 g.

Next, it was fractioned by agitation with 10 volumes of 90% phenol three times and the part remaining undissolved was washed with alcohol and ether thoroughly and dried. 200 mg. was the yield here.

It was taken up in 30 cc. of 10% NaCl, and shaken with many changes of an amylalcohol-chloroform mixture (1 : 4 by volume) to eliminate proteins according to Sevag and followingly dialyzed against running water for 2 days. The solution was further distilled in vacuo to 40 cc., and after addition of crystalline barium acetate to 10% and abs. alcohol to 20%, stood in a refrigerator for about 10 hours. The deposit here was centrifuged off, washed with alcohol and ether and dried. Dissolution in 50 cc. of a 10% solution of crystalline barium acetate, addition of alcohol to 20% and the subsequent operations were repeated four times. Crop, 120 mg. It was Neuberg-Saneyoshi- and Goldschmiedt-positive and contained hydrolyzable sulfur (detected as barium sulfate after acid hydrolysis) but was Molisch- and biuret-negative.

110 mg. of it were dissolved in 40 cc. of water, passed through an Amberlite IR-120 (30×40 mesh) column (2×30 cm.), and the effluent was distilled in vacuo to 15 cc., added to with 0.3 g. of crystalline sodium acetate and 5 volumes of alcohol (99%) and stood for 10 hours in a refrigerator. The centrifugate was washed with alcohol (99%) several times and with ether and dried in vacuo over $CaCl_2$—**Prep. I**. Yield, 75 mg.

Properties of the preparation

Electrophoresis. Prep. I was freed from sodium by the aid of Amberlite IR-120 and the syrupy condensate was precipitated with an alcohol-ether mixture (1 : 1 by volume) and dried in vacuo over $CaCl_2$. The substance given showed a single boundary after electrophoresis in phosphate buffer

pH 7.7, I 0.2 (Fig. 1).

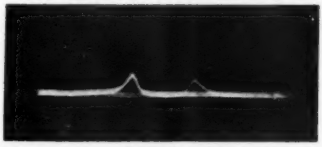

Fig. 1. Electrophoretic pattern (4/5×) of a 0.8% solution of Prep. I in phosphate buffer pH 7.7, I 0.2; current 10 mA; temperature 15°C. Exposure 1800 seconds after starting current. Mobility:

$$\frac{(-1.30)\times 0.0104\times 0.3}{0.01\times 1800}=-21.4\ \mathrm{cm^2/volt/sec}\times 10^{-5}$$

Paper partition chromatography of the sugar components. 5 mg. of the substance free from sodium were hydrolyzed in 0.5 cc. of HCl for 15 hours and paper-chromatographed after expelling off of HCl as described in the foregoing report.[2] Irrigation was continued for 70 hours. Spots were indicated at the positions corresponding to galacturonic and glucuronic acids and galactosamine (Fig. 2).

Fig. 2. Sugar chromatogram (2/15×) of a hydrolysate of Prep. I developed by descending irrigation with solvent butanol-pyridine-water (5:3:2). Temperature and duration of test 24°C and 70 hrs. respectively. Indicator aniline hydrogen phthalate reagent of Partridge. The standard solution was 1% with respect to individual sugars (hexuronic acids and hexosamine hydrochlorides) and 0.025 cc. was applied.

a: reference run. *b*: the hydrolysate of Prep. I. *1*, galacturonic acid. *2*, glucuronic acid. *3*, galactosamine. *4*, glucosamine.

Analyses. Analytical figures of Prep. I (sodium salt mixture) shown in Table I nearly coincide with the corresponding figures of the corresponding products prepared after alkaline extraction of pig skin in the foregoing work[2] (Table I of that report).

TABLE I

Composition of Prep. I

Analysis	In per cent	In equivalents per equivalent weight
Nitrogen*	2.73	1.08
Galactosamine†	32.19	1.0
Glucuronic acid‡	14.91	0.40
Galacturonic acid§	20.21	0.58
Hydrolyzable sulfur//	5.58	0.97
Ash (Na_2SO_4)¶	26.0	2.04

* Micro Kjeldahl. † A minor modification of Blix method.[6] ‡ Masamune and Aizawa.[7] § Masamune, Sakamoto & Aizawa.[8] // Suzuki.[9] ¶ Pregl.

Optical rotation in water

$$[\alpha]_D^{25}=\frac{-0.399\times 100}{1\times 0.71}=-56.2^\circ$$

The expenses of this work were defrayed by a grant given from the Ministry of Education through the Grant Committee for Scientific Researches. H. Masamune.

References

1) Meyer, Discussions Faraday Soc., 1953, **13**, 271.
2) Aizawa, Tohoku J. Exp. Med., this volume, p. 375.
3) Masamune, Conférences et rapports présentés au 3ème congrès international de biochimie, Bruxelles, 1955, Academic Press, New York, 1956, p. 72.
4) Masamune & Osaki, Tohoku J. Exp. Med., 1943, **45**, 121.
5) Masamune, *ibid.*, 1949, **50**, 107.
6) Masamune & Yosizawa, *ibid.*, 1956, **64**, 53.
7) Masamune & Aizawa, *ibid.*, this volume, p. 359.
8) Masamune, Sakamoto & Aizawa, *ibid.*, this volume, p. 367.
9) Suzuki, J. Bioch. (Japan), 1938, **27**, 367.

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Influence of Nutrition on Experimental Mouse Ectromelia Virus Infection

(The Effect of Varied Protein Intake on Experimental Mouse Ectromelia Virus Infection)

By

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(Received for publication, August 13, 1956)

Since the effectiveness of high protein diet in treatment of cirrhosis of the liver was reported by Patek & Post (1941)[1], a high protein diet commonly is used in the treatment of viral hepatitis. Proceeding their work, there were reports that a high protein diet was of benefit in preventing experimental dietary liver necrosis and fatty liver, and experimental toxic liver damage due to substances such as chloroform[2),3)], salvarsan[4)] and carbon tetrachloride[5)]. These experiments, of course, are not directly applicable to infectious hepatitis and other types of viral hepatitis. The virus of human infectious hepatitis is not pathogenic for experimental animals, but mouse ectromelia virus produces pathological changes in the liver similar to those seen in human infectious hepatitis. In the experiments to be described, the effect of dietary intake on survival was determined in mice infected with ectromelia virus.

Experimental

Methods

Experiment I

(a) Forty male mice of dd strain with an average weight of 16 g. were fed high protein and low protein diets for fifty-six days.

(b) Sixty-two male mice of sm strain with an average weight of 17 g. were fed high protein, low protein and low protein plus 0.8% methionine diets for thirty-seven days.

Upon completion of the above dietary regimens, all mice were inoculated intraperitoneally with 0.01 ml. of 1 : 10 dilution of ectromelia virus suspension per g. of body weight.

Survival period and survival rate after inoculation were determined by checking the cages for dead mice every twelve hours.

Experiment II

(a) Seventy-three male mice of dd strain with an average weight of 19.3 g. were fed high protein, low protein, low protein plus 0.8% methionine, and low protein plus alpha-tocopherol diets for forty-nine days. Alpha-tocopherol was added twice weekly in the amount of 10 mg. per mouse.

(b) Sixty male mice of sm strain with an average weight of 18.1 g. were fed high protein, low protein and low protein plus 0.8% methionine diets for thirty-eight days.

Upon completion of the above dietary regimens, all mice were inoculated intraperitoneally with 0.01 ml. of $1:10^9$ dilution of ectromelia virus suspension per g. of body weight.

Survival period and survival rate after inoculation were determined by checking the cages for dead mice every twelve hours.

In all experiments, the mice were kept at enviromental temperatures of 15–25°C.

The composition of the experimental diets was as follows.

The high protein diet consisted of casein 30%, sucrose 55%, lard 5%, cod liver oil 3% and Osborne-Mendel salt mixture 7%. The low protein diet consisted of casein 5%, sucrose 80%, lard 5%, cod liver oil 3% and Osborne-Mendel salt mixture 7%. To each diet was added 0.2 g. of Japanese brewer's yeast per mouse daily.

The mice were caged in groups of five and 15 g. of diet were presented daily to each group.

Ectromelia virus of the Sendai strain, maintained by serial intraperitoneally transfer in mice, was obtained from the Microbiological Institute. The livers of infected mice were suspended in isotonic saline containing 1,000 units penicillin and 1.0 mg. streptomycin per cc. and the resultant suspensions were centrifuged at 3,000 r.p.m. for 30 minutes.

Experimental mice were infected by intraperitoneal injection of 0.01 ml/g of diluted supernatant. The concentration of supernatant was 1:10 in Experiment I and in Experiment II was $1:10^9$.

Results

Experiment I

Food intake and weight gain. (a) The average daily food intake of both dietary groups was nearly the same, 2.53 g. for the high protein diet group and 2.43 g. for the low protein diet group. On the other hand, average body weight after fifty-six days of diet increased 6.04 g. in mice on the high protein diet but only 0.31 g. for animals on the low protein regimen. This difference was statistically significant at the less than 0.01 level.

(b) The average daily food intake of the high protein, the low pro-

tein and the low protein plus methionine diet groups were nearly the same. The average body weight after thirty-seven days, however, increased 8.14 g. in the high protein diet group, and decreased 1.34 g. and 3.89 g. in the low protein and in the low protein plus methionine groups. The difference between the high protein diet group and the two low protein diet groups was statistically significant.

Days survival after inoculation. In experiment (a), the average survival after inoculation was 3.94 days in the high protein diet group and 3.55 days in the low protein diet group, this difference being statistically significant at the less than 0.01 level (Fig. 1). In experiment (b), the average

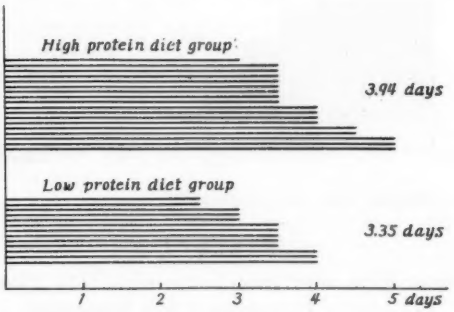

Fig. 1. Survival days after inoculation.

survival after inoculation was 3.48 days in the high protein diet group, 2.96 days in the low protein diet group and 2.85 days in the low protein plus methionine diet group. The difference between the high protein diet group and the remaining two diet groups was statistically significant (Fig. 2).

Survival rate after inoculation. In experiment (a), the survival rate of the high protein diet group was 94.4% 3 days after inoculation compared with 61.5% for the low protein diet group. This difference was statistically significant at the 0.034 level, but differences in survival rates at 2.5, 3.5, 4, and 4.5 days after inoculation were not statistically significant (Table I. and Fig. 3). In experiment (b), the survival rate 2.5 days after inoculation was 100% for the high protein diet group, 66.7% for the low protein diet group, and 46.1% for the low protein plus methionine diet group. The difference between the high and the two low protein diet groups was statistically significant (Table II and Fig. 4).

To summarize, in mice receiving relatively large doses (1 :10 dilution) of virus, the survival period was longer and the survival rate was higher for groups receiving the high protein diet than in animals receiving the low protein and the low protein plus methionine diets.

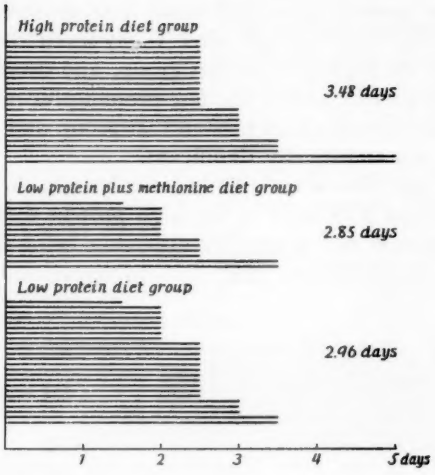

Fig. 2. Survival days after inoculation.

TABLE I

Survival Rate after Inoculation with 1:10 Dilution of Mouse Ectromelia Virus Suspension

Days after inoculation		2½	3	3½	4	4½
High protein diet for 56 days	Number of mice surviving	18	17	9	5	3
	Survival rate (%)	100	94.4	50.0	27.8	16.3
Low protein diet for 56 days	Number of mice surviving	11	8	3	0	0
	Survival rate (%)	84.6	61.5	23.1	0	0

Experiment II

Food intake and weight gain. (a) the average daily food intake was nearly the same for each group. Average body weight after thirty-seven days had increased 6.27 g. in the high protein diet group and 1.07 g. in the low protein plus methionine diet group, and had decreased 1.29 g. in the low protein diet group and 0.90 g. in the low protein plus alpha-tocopherol diet group. The difference between the high protein diet and the three low protein diet groups was statistically significant.

(b) The average daily food intake was nearly the same in each group. After thirty-eight days the average body weight had increased 5.39 g. in the high protein diet group and 0.43 g. in the low protein diet group,

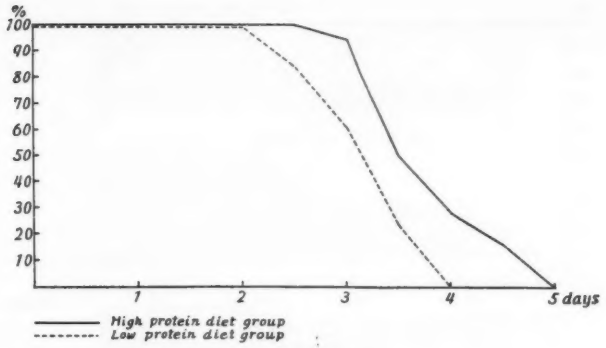

Fig. 3. Survival rate after inoculation.

TABLE II

Survival Rate after Inoculation with 1:10 Dilution of Mouse Ectromelia Virus Suspension

Days after inoculation		2	2½	3	3½	4
High protein diet for 37 days	Number of mice surviving	25	25	12	6	3
	Survival rate (%)	100	100	48	24	12
Low protein plus methionine diet for 37 days	Number of mice surviving	12	6	2	2	0
	Survival rate (%)	92.3	46.1	15.4	15.4	0
Low protein diet for 37 days	Number of mice surviving	23	16	5	2	0
	Survival rate (%)	95.8	66.7	20.8	8.3	0

whereas the average of the low protein plus methionine diet group has decreased 0.47 g. The difference between the high protein diet group and the two low protein groups was statistically significant.

Days survival after inoculation. In experiment (a), the average survival period after inoculation was 12.78 days for the low protein diet group, 12.00 days for the low protein plus alpha-tocopherol diet group, 11.88 days for the high protein diet group and 10.53 days for the low protein plus methionine diet group (Fig. 5). In experiment (b), the average survival period of the low protein plus methionine diet group was 11.69 days, that of the low protein diet group was 10.4 days and that of the high protein diet group was 9.03 days (Fig. 6).

Survival rate after inoculation. In experiment II (a), the survival rate

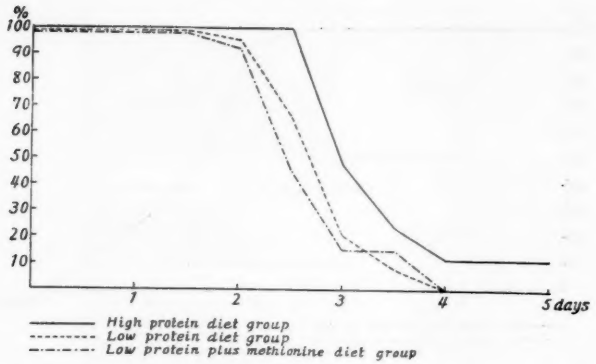

Fig. 4. Survival rate after inoculation.

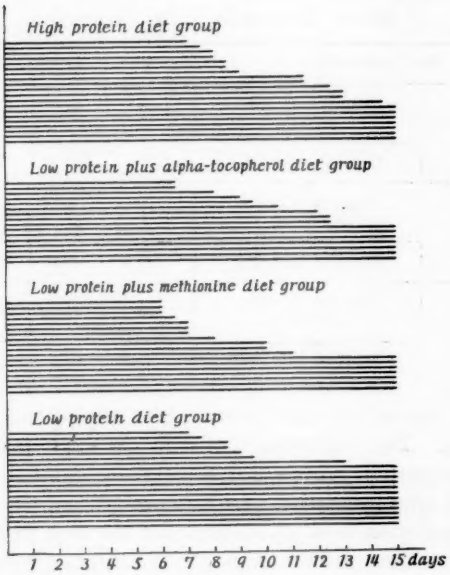
High protein diet group
Low protein plus alpha-tocopherol diet group
Low protein plus methionine diet group
Low protein diet group
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 days

Fig. 5. Survival days after inoculation.

after inoculation was nearly the same for each group, the difference between each group was not statistically significant. (Table III and Fig. 7).

In experiment II (b), the survival rate seven days after inoculation was 91% in the low protein plus methionine diet group, 90% in the low

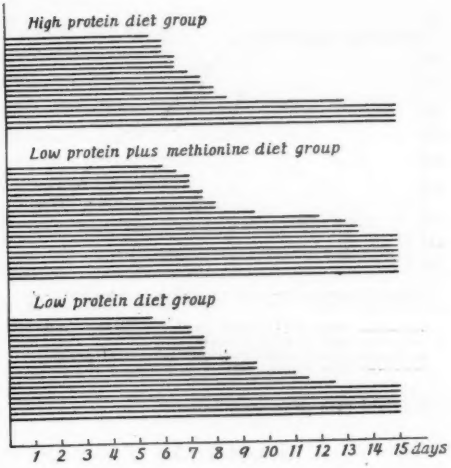

Fig. 6. Survival days after inoculation.

TABLE III

Survival Rate after Inoculation with $1:10^9$ Dilution of Mouse Ectromelia Virus Suspension

Days after inoculation		6	6½	7	7½	8	8½
High protein diet for 49 days	Number of mice surviving	20	20	20	19	18	16
	Survival rate (%)	100	100	100	95.2	90.1	80.0
Low protein plus methionine diet for 49 days	Number of mice surviving	18	15	14	11	11	10
	Survival rate (%)	100	83.3	77.8	61.1	61.1	55.6
Low protein plus alpha-tocopherol diet for 49 days	Number of mice svrviving	16	16	14	14	14	13
	Survival rate (%)	100	100	87.5	87.5	87.5	81.3
Low protein diet for 49 days	Number of mice surviving	19	19	19	18	17	17
	Survival rate (%)	100	100	100	94.8	89.4	89.4

protein diet group, and 61% in the high protein diet group. The difference between the high and the low protein diet groups was statistically significant at the 0.045 level (Table IV and Fig. 8).

To summarize, when the infecting dose of ectromelia virus was re-

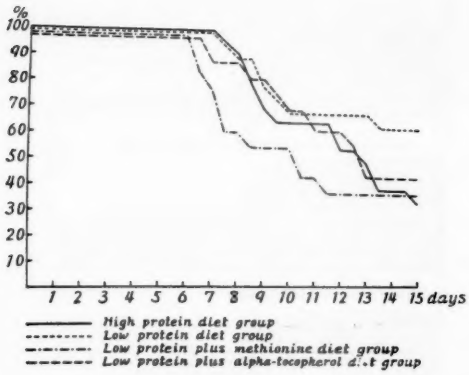

Fig. 7. Survival rate after inoculation.

TABLE IV

Survival Rate after Inoculation with $1:10^9$ Dilution of Mouse Ectromelia Virus Suspension

Days after inoculation		6	6½	7	7½	8	8½
High protein diet for 38 days	Number of mice surviving	17	14	11	10	8	6
	Survival rate (%)	94	77.8	61.1	55.6	44.4	44.3
Low protein plus methionine diet for 38 days	Number of mice surviving	22	21	20	17	15	13
	Survival rate (%)	100	95.4	90.9	77.3	68.2	59.1
Low protein diet for 38 days	Number of mice surviving	19	18	18	16	12	12
	Survival rate (%)	95	90	90	80	60	60

latively low ($1:10^9$ dilution) mice kept on a low protein diet were more resistant to ectromelia infection than animals fed a high protein diet.

Discussion

Concerning the relation of nutrition and experimental viral infection, it has been reported that malnutrition, and deficiency of thiamine, riboflavin, calcium-pantothenate, some inorganic salts, and tryptophane increase the resistance to experimental infection with the Lansing Virus of mice, Theiler's GD VII Virus, and various other viruses.[6),7),8),9)]

It is said that unlike bacteria and ricketsia, viruses may thrive best when the metabolism of experimental animals is active. On the other

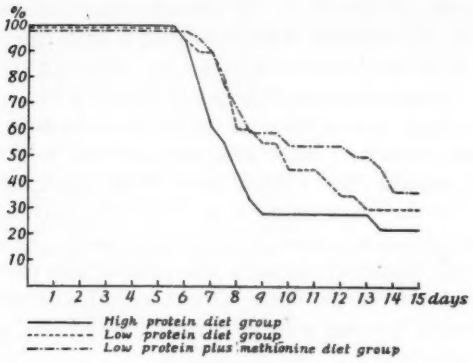

Fig. 8. Survival rate after inoculation.

hand, in thiamine deficiency, psittacosis in latent state was manifested[10] and the susceptibility of monkeys to intranasal infection with influenza virus A is increased.[11] The effect of dietary protein content likewise varies according to the experimental conditions and the virus employed. In experimental infection of mice with Lansing virus, a slight delay in onset of symptoms was seen in animals receiving a low protein (5% casein) diet.[12] On the other hand, neither a low protein diet (9% casein) nor a high protein diet (36% casein) exerted any discernible influence on infection with Theiler's GD VII virus in mice.[13]

Sprunt (1949)[14] reported that adult mice which were fed a low protein diet for two weeks were more resistant to infection with Swine influenza virus but this increased resistance to viral infection disappeared if the mice were kept on a low protein diet for a longer time, and immature mice were more susceptible to infection even though they were on the low protein diet.

Usually a considerable period of time is necessary to demonstrate decreased resistance by low protein diet in experimental infection,[6] and unlike bacterial infection, a low protein diet delays the onset of symptoms in experimental viral infections in short-term studies. With more prolonged studies, however, resistance to viral infection decreases just as in bacterial infection.

Our studies show that the size of the infecting dose of virus in an important factor in the relationship of nutritional factors to host resistance. With large doses of virus, both survival time and rate of survival were best in mice receiving a high protein diet. When small doses of virus were inoculated, however, the length of survival and the rate of survival were best in animals receiving low protein diets.

As mentioned previously, it is thought that viruses multiply most rapidly when the metabolism of the host is at a high rate, and conversely, multiplication of viruses is retarded by factors reducing the metabolic rate of the host. Thus, when small infecting doses of virus were employed, the lowered metabolic rate of mice on the low protein diets retarded growth of the virus, and therefore, these animals survived longer than those receiving a high protein diet. With large doses of virus, however, little time is needed for the virus to grow to lethal concentrations, and the retarding effect of a lowered metabolic rate is not seen. In fact, the survival rate was lower in animals receiving a low protein diet prior to inoculation of large doses of ectromelia virus. In these mice, decreased resistance due to depletion of protein reserves overshadowed the protective effects of lowered metabolic rate.

Summary

The effect of dietary protein content on length of survival and rate of survival was studied in mice infected with ectromelia virus. The mice were fed the experimental diets for five to eight weeks before inoculation of virus. In one group of experiments, small doses of virus were inoculated, in another group, large doses were employed.

With small doses of virus, both the length of survival and the rate of survival were best in mice receiving low protein diets. When large doses of virus were inoculated, however, both the survival time and the surviva rate was best in animals receiving a high protein diet.

It is postulated that with small doses of virus, the retarding effect of virus multiplication of lowered host metablism accounts for the increased survival of mice on low protein diets. With large doses of virus, this effect is overshadowed by decreased host resistance due to depleted protein reserves.

References

1) Patek & Post, J. Clin. Invest., 1941, **20**, 481.
2) Goldschmidt, Vars & Ravdin, *ibid.*, 1939, **18**, 277.
3) Miller & Wipple, A.J.M. Sci., 1940, **199**, 216.
4) Messinger & Hawkins, *ibid.*, 1940, **199**, 204.
5) Hove, Arch. Bioch., 1947, **17**, 467.
6) Clark, Ann. Rev. Microbiol., 1950, **4**, 353.
7) Foster, Johnes, Henle & Dorfman, J. Exp. Med., 1944, **79**, 221 ; *ibid.*, 1944, **80**, 257.
8) Kearney, Pond, Plass, Maddy, Elvehjem & Clark, J. Bact., 1948, **55**, 89.
9) Rasmussen, Waisman, Elvehjem & Clark, J. Inf. Dis., 1944, **74**, 41.
10) Rasmussen, Weaver, Elvehjem & Clark, Proc. Soc. Exp. Biol. & Med., 1952, **84**, 306.
11) Pinkerton, Nippon Rinsho (Jap.), 1954, **12**, 1.
12) Saslow, Wilson, Doan & Woolpert, J. Exp. Med., 1946, **84**, 113.

13) Jones, Foster, Henle & Alexander, Arch. Bioch., 1946, **11**, 481.
14) Maddy, Davies, Pond, Elvehjem & Clark, J. Inf. Dis., 1950, **86**, 202.
15) Sprunt, Fed. Proc., 1949, **8**, 366.
16) Ackerman, J. Exp. Med., 1951, **93**, 339.
17) Amano, Nippon Rinsho (Jap.), 1955, **13**, 1569.
18) Ruchman, J. Immunol., 1946, **53**, 51.
19) Clark, McClung, Pinkerton, Price, Schmider & Trager, Bact. Rev., 1949, **13**, 99.
20) Davies, Pond, Smith, Rasmussen, Elvehjem & Clark, J. Bact., 1952, **64**, 571.
21) Dohi, Virus (Jap.), 1952, **2**, 131.
22) Hukumi, Nippon Rinsho (Jap.), 1955, **13**, 1669.
23) Pond, Davies, Smith, Elvehjem, Rasmussen & Clark, J. Bact., 1952, **64**, 583.

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On the Measurement of the Respective Volumes of the Circulating Blood in Arteries and in Veins

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In the study of blood circulation it is important to know, in what ratio the circulating blood is distributed in arteries and in veins respectively, but at present there is no method to measure it, so we will report a theory, on which the respective volumes of the circulating blood in arteries and in veins may be measured.

Theoretical Considerations

When Va and Vv represent the volumes of the circulating plasma in arteries and in veins respectively, (Va+Vv), that is, the volume of the circulating plasma can be measured by dye T-1824. Therefore, if the other equation which includes Va and Vv can be obtained, the values of Va and Vv may be calculated, and using sodium para-aminohippurate (PAH), we could obtain such an equation.

Fig. 1 shows the relation of logarithm of the PAH concentration in the circulating plasma to time. C and c represent the PAH concentration in artery and in vein respectively, and the subscripts of C and c correspond to those of the time t. As shown in Fig. 1 PAH concentration in artery is always smaller than that in vein according to our experiments. In this study, we assume, that the PAH concentration in all arteries, as well as in all veins, shows the same value respectively at the same time.

As has been reported in the previous paper,[1] when t_0, t_2, t_3 and t_4 are respectively the time 5, 20, 30 and 50 minutes after intravenous injection of 2000 mg of PAH, logarithm of the PAH concentration in artery and in vein in the time interval (t_3, t_4) shows straight lines to the time, which run parallel to each other as in Fig. 1. And also in the time interval (t_0, t_2), logarithm of the PAH concentration in vein shows a straight line to the time as reported previously,[2] and according to our experiment, logarithm of the PAH concentration in artery in the same time interval (t_0, t_2) shows also a straight line, which runs parallel to that in vein. There-

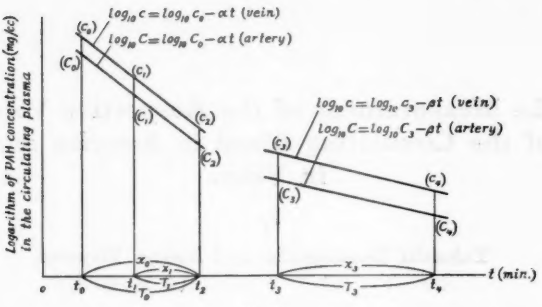
Logarithm of PAH concentration(mg/cc) in the circulating plasma
$\log_{10} c = \log_{10} c_0 - \alpha t$ (vein)
$\log_{10} C = \log_{10} C_0 - \alpha t$ (artery)
$\log_{10} c = \log_{10} c_3 - \beta t$ (vein)
$\log_{10} C = \log_{10} C_3 - \beta t$ (artery)
t (min.)

Fig. 1. Relation of logarithm of the PAH concentration in the circulating plasma in artery and in vein to time.

T_0, T_1 and T_3 represent the time from t_0 to t_2, from t_1 to t_2 and from t_3 to t_4 respectively, and x_0, x_1 and x_3 represent the volume of the PAH in the urine during the time T_0, T_1 and T_3 described above.

fore, when the direction coefficients of the lines, which logarithm of the PAH concentration in artery or in vein shows to the time in the time intervals (t_0, t_2) and (t_3, t_4), are $-\alpha$ and $-\beta$ respectively, the equations of such lines can be written as shown in Fig. 1.

The PAH in the urine excreted, for example, from the time g to h is the PAH excreted in the kidneys from the time ($g-t_{kb}$) to ($h-t_{kb}$), when t_{kb} represents the time needed for the PAH excreted in the kidneys to reach the bladder. In this report we call it k-b-time. In the time interval (t_3, t_4), when x represents the volume of the PAH in the urine excreted during time t, for example, from the time g to h, and y represents the volume of the PAH which flows from tissues into the circulating plasma from the time ($g-t_{kb}$) to ($h-t_{kb}$), equation (1) is obtained almost similarly as reported previously.[1),3)]

$$y = K_3 x \qquad (1)$$

K_3 in (1) is a constant, which is decided individually.

Similarly, in the time interval (t_0, t_2) also the following equation (2) can be obtained.

$$y = K_1 x \qquad (2)$$

K_1 is also a constant, which is decided individually.

When the whole volume of the PAH in the urine excreted till the time t_3 and t_4 are X_3 and X_4 respectively, the volume of the PAH in the tissues at the time (t_3-t_{kb}) and (t_4-t_{kb}) are Y_3 and Y_4 respectively, and the volume of the injected PAH is M, the following equations are obtained. The PAH concentration in artery and in vein at the time (t_3-t_{kb}), for example,

are $10^{\beta t_{kb}} \cdot C_3$ and $10^{\beta t_{kb}} \cdot c_3$ respectively.

$$10^{\beta t_{kb}} (C_3 V_a + c_3 V_v) = M - X_3 - Y_3 \quad (3)$$
$$10^{\beta t_{kb}} (C_4 V_a + c_4 V_v) = M - X_4 - Y_4 \quad (4)$$

Therefore,

$$10^{\beta t_{kb}} \{(C_3 - C_4) V_a + (c_3 - c_4) V_v\} = (X_4 - X_3) - (Y_3 - Y_4) \quad (5)$$

$(X_4 - X_3)$ in (5) is the volume of the PAH in the urine excreted in the time interval (t_3, t_4) and $(Y_3 - Y_4)$ is the volume of the PAH which flows into the circulating plasma from the tissues in the time interval $[(t_3 - t_{kb}), (t_4 - t_{kb})]$. When x_3 is the volume of the PAH in the urine excreted in the time interval (t_3, t_4), (6) is obtained from (1) and (5).

$$10^{\beta t_{kb}} \{(C_3 - C_4) V_a + (c_3 - c_4) V_v\} = x_3 - K_3 x_3 \quad (6)$$

In the time interval (t_0, t_2) also, when x_1 is the volume of the PAH in the urine excreted in the time interval (t_1, t_2), the following equation is similarly obtained.

$$10^{\alpha t_{kb}} \{(C_1 - C_2) V_a + (c_1 - c_2) V_v\} = x_1 - K_1 x_1 \quad (7)$$

From (6) and (7), (8) is obtained.

$$\{10^{\alpha t_{kb}} (C_1 - C_2) - 10^{\beta t_{kb}} (C_3 - C_4)\} V_a + \{10^{\alpha t_{kb}} (c_1 - c_2) - 10^{\beta t_{kb}} (c_3 - c_4)\} V_v = x_1 - x_3 - (K_1 x_1 - K_3 x_3) \quad (8)$$

In (8), if the relation of (9) exists,

$$x_1 = \frac{K_3}{K_1} x_3 \quad (9)$$

in other words, if, after deciding the time t_3, t_4 and t_2, the time t_1 which satisfies (9) can be determined, the following equation including only Va and Vv as unknown will be obtained. C_1 and c_1 are the PAH concentration in artery and in vein respectively at the time t_1, and x_1 is the volume of the PAH in the urine excreted in the time interval (t_1, t_2) and t_{kb} can be measured experimentally.

$$\{10^{\alpha t_{kb}} (C_1 - C_2) - 10^{\beta t_{kb}} (C_3 - C_4)\} V_a + \{10^{\alpha t_{kb}} (c_1 - c_2) - 10^{\beta t_{kb}} (c_3 - c_4)\} V_v = x_1 - x_3 \quad (10)$$

Fig. 2 shows a scheme of artery, capillary and vein. When C_3 is the PAH concentration in the artery at the time t_3, c'_3 is the PAH concentration in the vein when that blood reaches the vein through the capillary, and ΔC is the increase of the PAH concentration during that time, the following equation is obtained.

$$c'_3 = C_3 + \Delta C \quad (11)$$

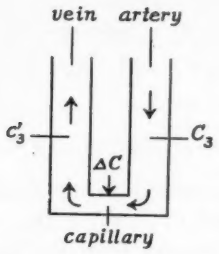

Fig. 2. A scheme of artery, capillary and vein.
C_3 represents the PAH concentration in artery at the time t_3, and c'_3 represents the PAH concentration in vein, when the arterial blood, the PAH concentration of which is C_3, reaches that vein passing through capillary, and ΔC represents the increase of PAH concentration during that time.

It is obvious, that ΔC will be proportional to the volume of the PAH, which flows into the circulating plasma from the tissues related to that capillary within the time Δt, which is needed for the blood to flow from the artery to the vein through the capillary, and it is also obvious, that the latter volume of the PAH will be also proportional to the volume of the PAH, which flows into the circulating plasma from the tissues in the whole body within the time Δt. Therefore (12) is obtained.

$$\Delta C = Q \cdot \Delta y \tag{12}$$

Q is a constant which is decided individually, and Δy is the volume of the PAH which flows into the circulating plasma from the tissues in the whole body within the time Δt.

When Δx is the volume of the PAH excreted in the kidneys within Δt, (13) is obtained from (1) and (12).

$$\Delta C = Q K_3 \cdot \Delta x \tag{13}$$

When p_3 is the excretion rate of PAH in the kidneys in the time interval (t_3, t_4), and v_3 is the renal plasma flow in the same time interval, Δx, that is, the volume of the PAH excreted in the kidneys within Δt at t_3 can be shown as (14).

$$\Delta x = p_3 v_3 \int_0^{\Delta t} C dt = p_3 v_3 C_3 \int_0^{\Delta t} 10^{-\beta t} dt = \frac{p_3 v_3 C_3 (1 - 10^{-\beta \Delta t})}{\beta \log 10} \tag{14}$$

From the equation shown in Fig. 1 (15) is obtained.

$$c'_3 = c_3 \cdot 10^{-\beta \Delta t} \tag{15}$$

And from (11), (13), (14) and (15), (16) is obtained.

$$c_3 \cdot 10^{-\beta \Delta t} = C_3 + \frac{QK_3 p_3 v_3 C_3 (1-10^{-\beta \Delta t})}{\beta \log 10} \tag{16}$$

When p_1 is the excretion rate of PAH in the time interval (t_0, t_2) and v_1 the renal plasma flow in the same time interval, (17) is similarly obtained.

$$c_2 \cdot 10^{-\alpha \Delta t} = C_2 + \frac{QK_1 p_1 v_1 C_2 (1-10^{-\alpha \Delta t})}{\alpha \log 10} \tag{17}$$

As Δt is considered to be extremely small, (18) is obtained from (16) and (17).

$$\begin{aligned} \frac{K_3}{K_1} &= \lim_{\Delta t \to 0} \frac{\beta C_2 (c_3 \cdot 10^{-\beta \Delta t} - C_3)(1-10^{-\alpha \Delta t}) p_1 v_1}{\alpha C_3 (c_2 \cdot 10^{-\alpha \Delta t} - C_2)(1-10^{-\beta \Delta t}) p_3 v_3} \\ &= \frac{C_2 (c_3 - C_3) p_1 v_1}{C_3 (c_2 - C_2) p_3 v_3} \end{aligned} \tag{18}$$

When T_3 is the time from t_3 to t_4, and T_0 from t_0 to t_2, x_3 and x_0, which are the volumes of the PAH in the urine excreted in the time interval (t_3, t_4) and (t_0, t_2) respectively, will be shown by (19) and (20).

$$x_3 = p_3 v_2 \int_0^{T_3} C dt = \frac{10^{\beta t_{kb}} p_3 v_3 C_3 (1-10^{-\beta T_3})}{\beta \log 10} \tag{19}$$

$$x_0 = p_1 v_1 \int_0^{T_0} C dt = \frac{10^{\alpha t_{kb}} p_1 v_1 C_0 (1-10^{-\alpha T_0})}{\alpha \log 10} \tag{20}$$

From (19) and (20), (21) is obtained.

$$\frac{p_1 v_1}{p_3 v_3} = \frac{10^{\beta t_{kb}} \alpha x_0 C_3 (1-10^{-\beta T_3})}{10^{\alpha t_{kb}} \beta x_3 C_0 (1-10^{-\alpha T_0})} = \frac{10^{\beta t_{kb}} \alpha x_0 C_3 (1-10^{-\beta T_3})}{10^{\alpha t_{kb}} \beta x_3 C_2 (10^{\alpha T_0}-1)} \tag{21}$$

And from (18) and (21), (22) is obtained.

$$\frac{K_3}{K_1} = \frac{10^{\beta t_{kb}} \alpha x_0 (c_3 - C_3)(1-10^{-\beta T_3})}{10^{\alpha t_{kb}} \beta x_3 (c_2 - C_2)(10^{\alpha T_0}-1)} \tag{22}$$

If t_{kb} is measured experimentally, x_1 which satisfies the relation of (9) can be calculated from (9) and (22), when x_0, that is, the volume of the PAH in the urine in the time interval (t_0, t_2) is known.

When T_1 is the time from t_1 to t_2 in the time interval (t_0, t_2), and x_1 is the volume of the PAH in the urine in the time interval (t_1, t_2), (23) is obtained similarly.

$$\frac{K_3}{K_1} = \frac{10^{\beta t_{kb}} \alpha x_1 (c_3 - C_3)(1-10^{-\beta T_3})}{10^{\alpha t_{kb}} \beta x_3 (c_2 - C_2)(10^{\alpha T_1}-1)} \tag{23}$$

From (9) and (23), (24) is obtained.

$$\frac{10^{\beta t_{kb}} \alpha (c_3 - C_3)(1-10^{-\beta T_3})}{10^{\alpha t_{kb}} \beta (c_2 - C_2)(10^{\alpha T_1}-1)} = 1 \tag{24}$$

Therefore

$$T_1=\frac{1}{\alpha}\log_{10}\{1+\frac{10^{\beta t_{kb}}\alpha(c_3-C_3)}{10^{\alpha t_{kb}}\beta(c_2-C_2)}(1-10^{-\beta T_3})\} \qquad (25)$$

The time t_1, which satisfies the relation of (9), is determined from (25), namely, t_1 is equal to (t_2-T_1).

When C_1 and c_1 are the PAH concentration in artery and in vein at t_1, (10) is obtained as mentioned above, and when a is the volume of the circulating plasma measured with dye T-1824, the following equation is obtained.

$$Va+Vv=a \qquad (26)$$

Va and Vv, that is, the volumes of the circulating plasma in arteries and in veins can be calculated from (10) and (26).

This is the theory of measuring the volumes of the circulating plasma in arteries and in veins respectively, and we intended to know the k-b-time in approximation by measuring the time, which would be needed for indigocarmin injected intravenously to appear in the bladder, but due to refusal of test persons we could not perform it. According to Smith *et al*,[4] total delay time, that is, the time needed for hippuran to reach the bladder from the antecubital vein was two minutes in one case, and total delay time is thought not to differ extremely from k-b-time, so we tried to use two minutes as k-b-time in calculation, but, as k-b-time was assumed to be two minutes in calculation, many cases showed negative values of Va. And the values of Va and Vv show considerably large variation due to small increase or decrease of k-b-time. Therefore, it is necessary to measure the accurate k-b-time in order to get the volumes of the circulating plasma in arteries and in veins. It is natural that k-b-time or total delay time will vary in different individuals and with the same individual under different circumstances. As stated above, we could not measure k-b-time actually, so in order to examine, whether our theory would be right or not, we calculated the upper and lower limits of k-b-time, at which both Va and Vv showed positive values in calculation, as follows:

i) When Va=o and Vv=a,
then (10) becomes as follows:

$$\{10^{\alpha t_{kb}}(c_1-c_2)-10^{\beta t_{kb}}(c_3-c_4)\}a=x_1-x_3 \qquad (27)$$

As $c_1=c_2\cdot 10^{\alpha T_1}$ is evident from the equation shown in Fig. 1,

$$c_1-c_2=c_2(10^{\alpha T_1}-1) \qquad (28)$$

On the other hand, from (24)

$$10^{\alpha T_1}-1=\frac{10^{\beta t_{kb}}\alpha(c_3-C_3)}{10^{\alpha t_{kb}}\beta(c_2-C_2)}(1-10^{-\beta T_3}) \tag{29}$$

and from (9) and (22)

$$x_1=\frac{10^{\beta t_{kb}}\alpha(c_3-C_3)(1-10^{-\beta T_3})}{10^{\alpha t_{kb}}\beta(c_2-C_2)(10^{\alpha T_0}-1)}x_0 \tag{30}$$

so we get (31) from (27), (28), (29) and (30).

$$\left\{\frac{\alpha(c_3-C_3)(1-10^{-\beta T_3})c_2}{\beta\ (c_2-C_2)}-(c_3-c_4)\right\}a=\frac{\alpha(c_3-C_3)(1-10^{-\beta T_3})x_0}{10^{\alpha t_{kb}}\beta(c_2-C_2)(10^{\alpha T_0}-1)}$$
$$-\frac{x_3}{10^{\beta t_{kb}}} \tag{31}$$

From (31) we can get the value of k-b-time, at which Va=o and Vv=a.

ii) When Va=a and Vv=o, we can get the following equation similarly.

$$\left\{\frac{\alpha(c_3-C_3)(1-10^{-\beta T_3})C_2}{\beta\ (c_2-C_2)}-(C_3-C_4)\right\}a=\frac{\alpha(c_3-C_3)(1-10^{-\beta T_3})x_0}{10^{\alpha t_{kb}}\beta(c_2-C_2)(10^{\alpha T_0}-1)}$$
$$-\frac{x_3}{10^{\beta t_{kb}}} \tag{32}$$

From (32) we can get the value of k-b-time, at which Va=a and Vv=o. And between the respective k-b-times calculated from (31) and (32), Va and Vv show both positive values in calculation.

Experimental Method

About 25 male adults we made our experiments. They were given water properly by mouth several times from 30 minutes prior to the start of experiment till the end of it, so as to equalize the urine excretion per minute. Blood samples and urine specimen were collected as follows: after intravenous injection of 2000 mg of PAH blood was taken from the femoral artery, as well as from the antecubital vein, three times respectively, in the time intervals (t_0, t_2) and (t_3, t_4), and the whole urine was collected between 7 and 22 minutes and between 32 and 52 minutes after PAH injection. Immediately after these procedures dye T-1824 was injected into the vein, and venous blood was taken 10 minutes later. PAH concentration in the plasma, the whole volume of the PAH in the urine and the volume of the circulating plasma were measured or calculated, and a graph to show the relation of logarithm of the PAH concentration in the plasma to time was made from the measured values. Calculation was done on 18 cases, in which logarithm of the PAH concentration in artery and in vein showed, to the time, two straight lines running parallel to each other.

The urine was collected spontaneously, and PAH concentration was measured by the method of Smith *et al.*[5]

TABLE I

Range of k-b-time, in Which Va and Vv Show Positive Values, and Its Mean Value

k-b-time represents the time needed for the PAH excreted in the kidneys to reach the bladder.

Case	Age	Diagnosis	Range of k-b-time (min.)	Mean k-b-time (min.)
1	35	Normal	6.5——7.5	7.0
2	40	"	1.8——2.9	2.4
3	43	"	4.8——5.6	5.2
4	54	"	1.7——2.8	2.3
5	26	"	4.5——5.3	4.9
6	29	"	3.7——4.6	4.2
7	55	"	4.5——5.5	5.0
8	45	"	6.4——7.0	6.7
9	38	"	1.6——2.7	2.2
10	62	"	0.3——1.2	0.8
11	41	"	3.1——4.2	3.7
12	57	Essential hypertension	3.9——5.0	4.5
13	58	"	0.1——1.0	0.6
14	59	"	5.8——6.5	6.2
15	44	"	5.2——5.5	5.4
16	65	"	4.0——4.6	4.3
17	54	"	0.8——1.3	1.1
18	53	"	8.8——9.7	9.3
		Average		4.2

Experimental Results and Criticism on the Theory

The range of k-b-time, in which both Va and Vv show positive values in calculation, and the mean value of it are shown in Table I.

The mean values are between 0.6 and 9.3 minutes and the average is 4.2 minutes. This average time is in accordance with the hitherto reported value of the time needed for intravenously injected indigocarmin to appear first in the bladder in normal persons, and so it is also thought to be in accordance with the actual k-b-time. From this it is presumed that our theory may be right. Case 18 is in advanced stage of essential hypertension, and so it is presumed that the renal lesion in this case will be severe and therefore the actual k-b-time will be greater than normal. And as shown in Table I the calculated k-b-time in this case is considerably

greater than in others, and this fact is thought to support the theory too.

So, if k-b-time is measured actually, the true volumes of the circulating plasma in arteries and in veins may be calculated. Whether this expectation will be right or not, will be solved by calculation after the actual measurement of k-b-time.

Fig. 3

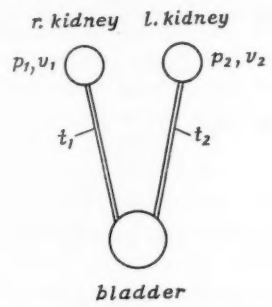

Fig. 4

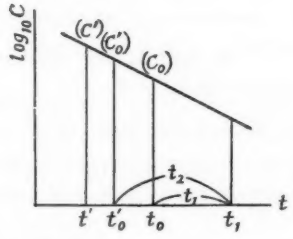

Fig. 3. A scheme of both kidneys and bladder.
p_1 and v_1 represent the excretion rate of PAH and the renal plasma flow in the right kidney, and p_2 and v_2 represent those in the left kidney. t_1 and t_2 represent the time needed for the PAH excreted in the right and left kidney to reach the bladder respectively.

Fig. 4. Relation of logarithm of the PAH concentration in artery to time.

There is a problem to be answered. It is that there are two kidneys and each k-b-time will be different. When, as in Fig. 3, the excretion rate of PAH, renal plasma flow and k-b-time are p_1, v_1 and t_1 respectively concerning the right kidney, and p_2, v_2 and t_2 concerning the left ($t_1 < t_2$), and as in Fig. 4 C_0 is the PAH concentration in artery at the time t_0, C_0' is that at the time t_0' and the time from t_0' to t_1 is t_2, dx_1 and dx_2, which are the volumes of the PAH, which, at the time t_1, reaches the bladder from the right and left kidney respectively within a minimum time dt, will be shown as follows (letters in equations in this chapter have no relationship with those used in the chapter of Theoretical Considerations) :

$$dx_1 = p_1 v_1 C_0 dt \qquad (1)$$
$$dx_2 = p_2 v_2 C_0' dt \qquad (2)$$

When $-\alpha$ is the direction coefficient of the straight line which logarithm of the PAH concentration in artery makes to the time,

$$C_0' = C_0 \cdot 10^{\alpha(t_2 - t_1)}$$

and so (2) becomes as follows:

$$dx_2 = p_2 v_2 C_0 \cdot 10^{\alpha(t_2-t_1)} dt \qquad (3)$$

Therefore, the volume of the PAH dx, which reaches the bladder from both kidneys at the time t_1 within dt, can be shown as follows:

$$dx = dx_1 + dx_2 = \{p_1 v_1 + p_2 v_2 \cdot 10^{\alpha(t_2-t_1)}\} C_0 dt \qquad (4)$$

When v is the renal plasma flow and the following relations exist,

$$v_1 = k_1 v$$
$$v_2 = k_2 v$$

(k_1 and k_2 are constant and $k_1 + k_2 = 1$)

(4) becomes as follows:

$$dx = \{p_1 k_1 + p_2 k_2 \cdot 10^{\alpha(t_2-t_1)}\} v C_0 dt \qquad (5)$$

In (5) $\{p_1 k_1 + p_2 k_2 \cdot 10^{\alpha(t_2-t_1)}\}$ is constant and can be represented as p (constant), so (5) becomes as follows:

$$dx = p v C_0 dt \qquad (6)$$

Equation (6) represents the volume of the PAH which reaches the bladder from both kidneys at the time t_1 within dt, when p, C_0 and t_1 are the excretion rate of PAH at t_0, the PAH concentration in artery at t_0 and k-b-time respectively. In other words, when both kidneys are considered as a single organ, it suffices to measure the lesser k-b-time concerning either the right or left kidney.

Another problem is, that, though p is so decided that dx in (6) is shown by using C_0, dx can not only be shown by using C_0, but it can also be shown by using C′ which is the PAH concentration in artery at an arbitrary time t′ prior to t_1, as follows:

$$dx = p' v C' dt \qquad (7)$$

(p′ is a constant)

In other words, it can also be thought, that the PAH excreted in the kidneys at an arbitrary time t′, namely, at an arbitrary PAH concentration C′ in artery reaches the bladder at t_1, and the k-b-time is $(t_1 - t')$. As t′ is arbitrary, $(t_1 - t')$ is not constant even in the same individual. Therefore, k-b-time may be thought unnecessary to be measured actually, and the PAH concentration C′ in artery, at which PAH is excreted in the kidneys, may be thought also to be arbitrary. Though this reasoning may be right in discussion in this chapter, it can not be applied to each equation described in the chapter of Theoretical Considerations. It can be easily proved, and though the proof is omitted in this report, actually measured k-b-time should be always taken into consideration.

The last problem is the definition of the circulating blood. Blood is composed of the circulating and depot blood. The so-called depot blood, however, is not completely cut off from circulation, but circulates, even if very slowly, and concerning the circulating blood, on the other hand, it does not circulate with the same velocity even in the same part of an artery or a vein. So, the blood circulation is considered to take place in combination of various velocities of blood from extremely slow to very fast. Therefore, the precise definition of the circulating blood is not clear at the present time. In the present experiment, however, the volume of the circulating plasma was measured, following conventional way, at the blood sample, which was taken ten minutes after the intravenous injection of dye T–1824.

For applying this theory to the measurement of the volumes of the circulating plasma in arteries and in veins, it is necessary, that the respective volumes of the circulating plasma in them, mode of water exchange between blood and tissues, renal plasma flow and urine excretion per minute are kept almost constant during the experiment. Considering the possible change of the renal plasma flow, we differentiated it as v_1 and v_3 in the time intervals (t_0, t_2) and (t_3, t_4) respectively, but v_1 and v_3 must be kept almost constant in the corresponding intervals. As the excretion rate of PAH would differ naturally with the PAH concentration in artery, it was differentiated as p_1 and p_3 in the time intervals (t_0, t_2) and (t_3, t_4) respectively, but for convenience, p_1 and p_3 were assumed to be constant in the respective intervals.

Theoretically, k-b-time can be measured by subtracting the time needed for PAH to reach the renal artery from the antecubital vein, from the time needed for PAH to reach the bladder from the antecubital vein, and the former time may be substituted by the circulation time from the antecubital vein to the femoral artery, measured with the method of Blumgart *et al*[6] for example, and the latter by the value measured with indigocarmin. The appearance of urine from ureters into bladder, however, is not continuous, but intermittent, and therefore the measured k-b-time and consequently the calculated volumes of Va and Vv will be accompanied with some error.

In this theory the PAH concentration in the renal veins is considered to be the same as that in other veins, but practically the former is lesser than the latter, so some correction will be needed for the values of Va and Vv. In the above mentioned theory the circulating plasma in arteries includes that in the heart and in the lungs, because, as reported previously[1], the volume of the extracellular fluid in the lungs is thought to be very small and migration of PAH from the lung tissue into the plasma during the blood circulation through pulmonary vessels is considered to be very

little, and therefore the PAH concentration in the circulating plasma in the heart and in the lungs can be regarded as same as that in arteries.

The reason, why we intended to try to measure the volumes of the circulating blood in arteries and in veins, consists in comparing the rate of blood distribution in arteries and in veins in hypertensive patients with that in normal persons, but in this present experiment without actual measurement of k-b-time, that is, the time needed for the PAH excreted in the kidneys to reach the bladder, it is of course impossible.

Conclusion

The theory of measuring the volumes of the circulating blood in arteries and in veins respectively was reported, and from the results of the experiment on this theory it is presumed that the theory may be right. And in order to get their values it is necessary to measure actually the time needed for the PAH excreted in the kidneys to reach the bladder.

References

1) Yamaguchi & Hino, Tohoku J. Exp. Med., 1955, **62**, 243.
2) Yamaguchi, *ibid.*, 1954, **60**, 209.
3) Kishine, Igarashi, Tsukamoto, Suto & Watanabe, *ibid.*, 1952, **56**, 275.
4) Smith, Goldring & Chasis, J. Clin. Invest., 1938, **17**, 263.
5) Smith, Finkelstein, Aliminosa, Crawford & Graber, *ibid.*, 1945, **24**, 388.
6) Blumgart & Weiss, *ibid.*, 1927, **4**, 399.